# The kinetics and mechanisms of non-thermal inactivation of *L. monocytogenes* and *E. coli* in raw milk cheeses.

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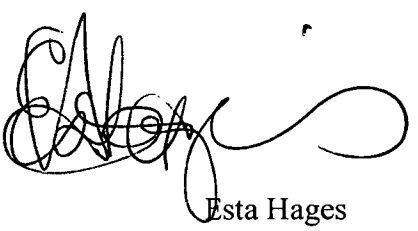

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**Abstract**

*Listeria monocytogenes* and *Escherichia coli* are food borne pathogens of concern to the Australian dairy industry. Recent consumer and producer requests to allow the manufacture and sale of raw milk cheeses in Australia have caused concern for the industry since raw milk has been identified as a vehicle for the transmission of these, and other, pathogens. Globally *L. monocytogenes* and *E. coli* have been associated with severe food-borne illness arising from the consumption of cheeses, demonstrating the ability of these organisms to survive despite the adverse environmental conditions imposed upon them in fermented milk products. An understanding of the ability of pathogens to survive the inimical conditions imposed by fermentation could contribute to the prevention of further outbreaks.

This thesis describes the influence of temperature on low pH- and low water activity-induced inactivation of *L. monocytogenes* and *E. coli* in cheese made from both raw and pasteurised mil and in an analogous broth system. In addition, proteomic responses of *L. monocytogenes* to progressive acidification such as occurs during fermentation, were undertaken.

The influence of temperature, that is not of itself lethal (i.e. in the range of 4 - 45°C), on the survival of *L. monocytogenes* under growth-preventing pH and water activity conditions was investigated. Bacterial inactivation under these conditions is referred to, in this thesis, as non-thermal inactivation. Inactivation rates for three strains of *L. monocytogenes* were determined in rich broth adjusted to pH 3.5 and water activity 0.90, to prevent growth, and for temperatures in the range 5 to 45°C. Twenty eight inactivation rates were determined, plotted on Arrhenius coordinates (i.e. 1/[absolute temperature] *vs. ln*[rate]), and lines of best fit determined by simple linear regression. The inactivation rate responses were comparable to those reported elsewhere, namely for i) *E. coli* inactivation in a model salami product and analogous broth system (McQuestin et al. 2006); ii) *E. coli* and *L. monocytogenes* inactivation in broth adjusted to pH 3.5 and water activity 0.90 (Zhang et al. 2010);and iii) *E. coli* inactivation in a variety of fermented meat products and analogous systems (McQuestin et al. 2009). The results showed that non-lethal temperature is also a strong determinant of the rate of inactivation of *L. monocytogenes* in environments in

which growth is prevented by pH and water activity. To extend these studies, a meta-analysis of 45 independent studies was undertaken to investigate the relative influence of pH, water activity, and temperature on *L. monocytogenes* survival. Published data were re-evaluated to determine rates of inactivation, providing 1195 data over a pH range from 2.7 to 7.4, $a_w$ range from 0.793 to 0.99, temperature range from 0-68°C, and in various food products, and for various processes and *L. monocytogenes* strains. When the data were presented as an Arrhenius model, temperature (0-42°C) accounted for 25% of the variance in *ln*(inactivation rate) data. The pH or water activity measured in broth or in food accounted for 21% and 6% respectively, of the variability in the data.

The inactivation kinetics of *L. monocytogenes* and *E. coli* in cheese, when introduced as either contaminants in raw milk, or as post-pasteurisation contaminants in pasteurised milk used for cheese making, were evaluated. Raw and pasteurised semi-hard Manchego style cow's milk cheeses were prepared with milk inoculated with either *L. monocytogenes* Scott A or *E. coli* M23 (an acid tolerant non-pathogenic strain) prepared in both stationary and exponential stages of growth. The final pH of the cheese was 4.5 to 4.7 and final $a_w$ 0.91 to 0.92, conditions which, in combination, prevent the growth of both *L. monocytogenes* and *E. coli*. Cheeses were prepared and stored at six temperatures in the range 4°C to 25°C. Rates of change of microbial population density were determined and modelled using simple linear regression, where appropriate, and rates of change for both species plotted as a function of temperature using Arrhenius plots. Differences due to the physiological state of the cells (i.e., stationary or exponential growth phase) at the time of cheese making were also analysed. Consistent with the results of broth studies, temperature was shown to have the greatest effect on the rate of inactivation of both species of bacteria however the response was not the same as in broth.

A second cheese challenge trial was undertaken to ascertain the robustness to accidental contamination with bacterial pathogens of a raw sheep's milk, Roquefort-style, cheese. Cheeses were prepared under laboratory conditions from raw ewe's milk inoculated with three strains each of both *L. monocytogenes* and *E. coli*. Triplicate inoculated cheeses were matured at each of six temperatures in the range 4°C to 20°C. Pathogen inactivation rate was modelled using simple linear regression

and rates of changes for both species plotted as a function of temperature as an Arrhenius plot. All trials were followed for the equivalent of the normal commercial maturation time for the cheese so that the effect of pH changes in the cheese (specifically, pH returning to neutrality later in ripening as is typical in mould-ripened cheeses) could be assessed. Fat content, protein content, $a_w$ and pH were measured to ascertain the potential influence of these factors on the fate of the introduced pathogens in the cheese. The results confirmed that raw ewe's milk Roquefort-style cheese does not support the growth and survival of *L .monocytogenes* or *E. coli* and that inactivation of these pathogens occurs when conditions of pH and water activity become inimical for growth. The results show that the inactivation kinetics of *E. coli* and *L .monocytogenes* in raw ewe's milk Roquefort-style cheese are consistent with those observed in broth (but not those observed in semi-hard cheese) and support the hypothesis that vegetative bacterial pathogens in foods that prevent their growth are inactivated at a rate that increases with increasing temperature as hypothesised by Ross et al (2008). The results are not consistent, however, with one of the findings of Zhang et al (2010) who concluded that there was no systematic differences between either the non-thermal inactivation rates of *E. coli* and *L. monocytogenes* at temperatures in the range 5 to 40°C in inimical environments.

To begin to elucidate the physiology of such 'non-thermal' inactivation processes, the proteome changes in *L monocytogenes* in response to progressive acidification were studied in a model cheese fermentation system. Proteins synthesised by cultures subjected to conditions of pH decreasing from pH 5.0 to 4.6 over time were compared with proteins synthesised by stationary phase cultures grown under neutral pH conditions. In the initial growth stages, when pH was mild (pH 5.0) proteins commonly associated with cell growth were up-regulated. Proteins associated with stress responses (significantly, cold shock proteins, heat shock proteins and the Sigma B operon) were down regulated. As the cell culture continued to grow and the pH continued to decrease proteins associated with the acid-tolerance response and virulence (Sigma B operon, listeriolysin O, internalin B, F0F1 system, PrfA) were up-regulated to varying degrees. The use of proteomic techniques provided an overview of the physiology of *L. monocytogenes* subjected to low, and eventually inimical, pH and was consistent with current knowledge of acid-tolerance responses.

In all, the work presented in this thesis develops the present understanding of the response of *L. monocytogenes* and *E. coli* to non-lethal storage temperature and inimical conditions relevant to the manufacture of cheeses from both raw and pasteurised, milk. The results from Chapters 2 and 4 support the hypothesis that non-lethal temperature is a key factor governing the rate of inactivation of vegetative bacteria in foods when other hurdles prevent their growth but indicate that the temperature effect may not be independent of the effect of food type, bacterial species and bacterial strain.

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# Chapter 1 Introduction - Raw Milk Cheese and the non-thermal inactivation of *L. monocytogenes* and *E. coli*.

## 1.1 Fermented dairy products: Cheese

Most nations worldwide incorporate some form of fermented dairy product into their cuisine. Cheese is the generic name for one of the most common fermented dairy products encompassing over 500 different varieties made from the milk of cow, goat, sheep and buffalo. The original objective of cheese making was to preserve the nutritional content of perishable milk resources and dates back as far as 6000 BC (Fox et al. 2000). Cheese, however, is also a food of epicurean qualities with production ranging from small artisanal cheeseries through to large scale commercial factories.

Milk from all sources contains solids made up of two distinct types of proteins (caseins and whey proteins), milk fat, lactose, citric acids and ash (mineral salts associated with casein) (Johnson 1998). The composition of milk is affected not only by species but also by an animal's breed, genetics, feed, living conditions, stage of lactation and its health, and is a factor governing the characteristics of the final product made from it. The development of the desired texture and pH is the first phase of cheese production and is controlled through milk composition and the rate and extent of acid development by starter cultures. The second phase of cheese production, commonly referred to as ripening, curing or maturation, encompasses the development of desired physical and chemical characteristics and is influenced by the first phase but is dominated by a number of chemical and enzymatic processes together with the metabolism of a number of microorganisms (Johnson 1998).

All cheeses, regardless of the manufacturing protocol, can be classified as soft, semi hard (semi soft), hard or very/extra hard depending upon their moisture content/casein ratio (Gripon 1997; Farkye 2004b). The classification is arbitrary but is the most common method used to systematically group together cheeses that are alike in the characteristics of body, consistency and compactness of the curd, all of which are directly determined by the moisture content (Farkye 2004b).

### 1.1.1 Coagulation

Cheese making is primarily a dehydration process. It begins with the formation of a coagulum where milk proteins are clotted, entrapping milk fat, water and water-soluble components (Johnson 1998). The coagulum is porous and can contract to expel liquid, a process termed syneresis (Gunasekaran and Ak 2003). The undisturbed coagulum expels moisture very slowly, which is why the coagulum is manipulated through cutting and stirring to increase the surface area and facilitate removal of moisture. Syneresis is also enhanced by the development of acid or an increase in temperature. In the cheese making process the development of acid together with cutting and stirring of the curd are processes that result in the greatest expulsion of moisture from the coagulated protein mass or curds (Johnson 1998; Gunasekaran and Ak 2003). At the same time the curd undergoes cooking which involves heating the curd (to between 38 and 55°C depending on the cheese type) and also facilitates contraction of the protein matrix and enhances syneresis (Gunasekaran and Ak 2003). Heating also increases acid production by starter cultures and together they affect the dissolution of calcium phosphate, which plays a role in final cheese characteristics (Gunasekaran and Ak 2003). The moisture component (commonly referred to as the whey) contains whey protein, the remaining milk lactose and other water-soluble components. It is removed and the remaining curds are formed into whatever shape is required by the type of cheese being made. The extent of syneresis is affected by; the firmness of the coagulum at cutting, the curd surface area, curd acidity, temperature, and milk composition and is specific to a particular cheese type (Walstra et al. 1999). Syneresis is manipulated by cheese makers to control moisture content, and microbial and enzymatic processes in the cheese and is, therefore, a factor that ultimately influences the ripening and quality of cheese (Gunasekaran and Ak 2003).

Three basic methods can be used to produce the curd. Each method involves development of acid, by a starter culture or direct acid application, and the concentration of milk solids by the expulsion of the whey (Johnson 1998). The three methods go by various names but are essentially cheese production by acid coagulation (acid set or acid curd), by enzyme coagulation (rennet set or rennet curd) or by concentration (heat-precipitated or heat/acid). Whilst agreement on a definitive

classification scheme for cheese does not exist at this time, the method of production has been proposed as a valid way to classify the many different types of cheese and an example of this classification is shown in Figure 1.1 (Davis 1965; Fox et al. 2000). Cheeses can also be classified dependent upon their final moisture content which is affected by the method of production and also by the method used to ripen the cheese. Figure 1.1 includes classifications based upon moisture and ripening method and serves to highlight the inherent 'complexity' to cheese classifications due to the many combinations of production method, moisture contents and ripening methods that may be employed for any given style of cheese.

Progressive acidification by starter cultures occurs in the production of the majority of cheese types. However, the curd of some varieties such as Mozzarella and Cottage cheese, are routinely acidified through the addition of acid or acidogen (such as acetic and lactic acid or gluconic acid-δ-lactone) instead of using starters. In the production of acid-coagulated cheeses, the acid produced by starter cultures (or acid added by cheese makers) causes milk caseins to gel when the pH reaches approximately 5.2. Casein micelles are unable to form aggregates at neutral pH due to their charge repulsion (Johnson 1998). In addition, the micelle cores are hydrophobic and are unable to interact with the *C*-terminal hydrophilic peptide regions of κ-caseins molecules that protrude from the cores into the aqueous phase, a process termed stearic repulsion (Johnson 1998). As the pH decreases, casein molecules begin to dissociate from their micelles due to the disintegration of calcium-phosphate complexes in the liquid milk (Johnson 1998). The charge on the molecules is also reduced, hydrophobicity is increased and the protruding portions of κ-caseins molecules are thought to retract into the micelle core (Johnson 1998). At approximately pH 4.95, a visible gel is formed by the aggregation of solubilised casein molecules and micelles (Johnson 1998). Cottage cheese gel is cut into small cubes at approximately pH 4.65-4.75 and cream cheese gel is not cut but rather stirred at pH 4.4-4.5 and the whey removed by centrifugation (Johnson 1998). The acid-coagulated varieties of cheese are consumed fresh and do not undergo a ripening period.

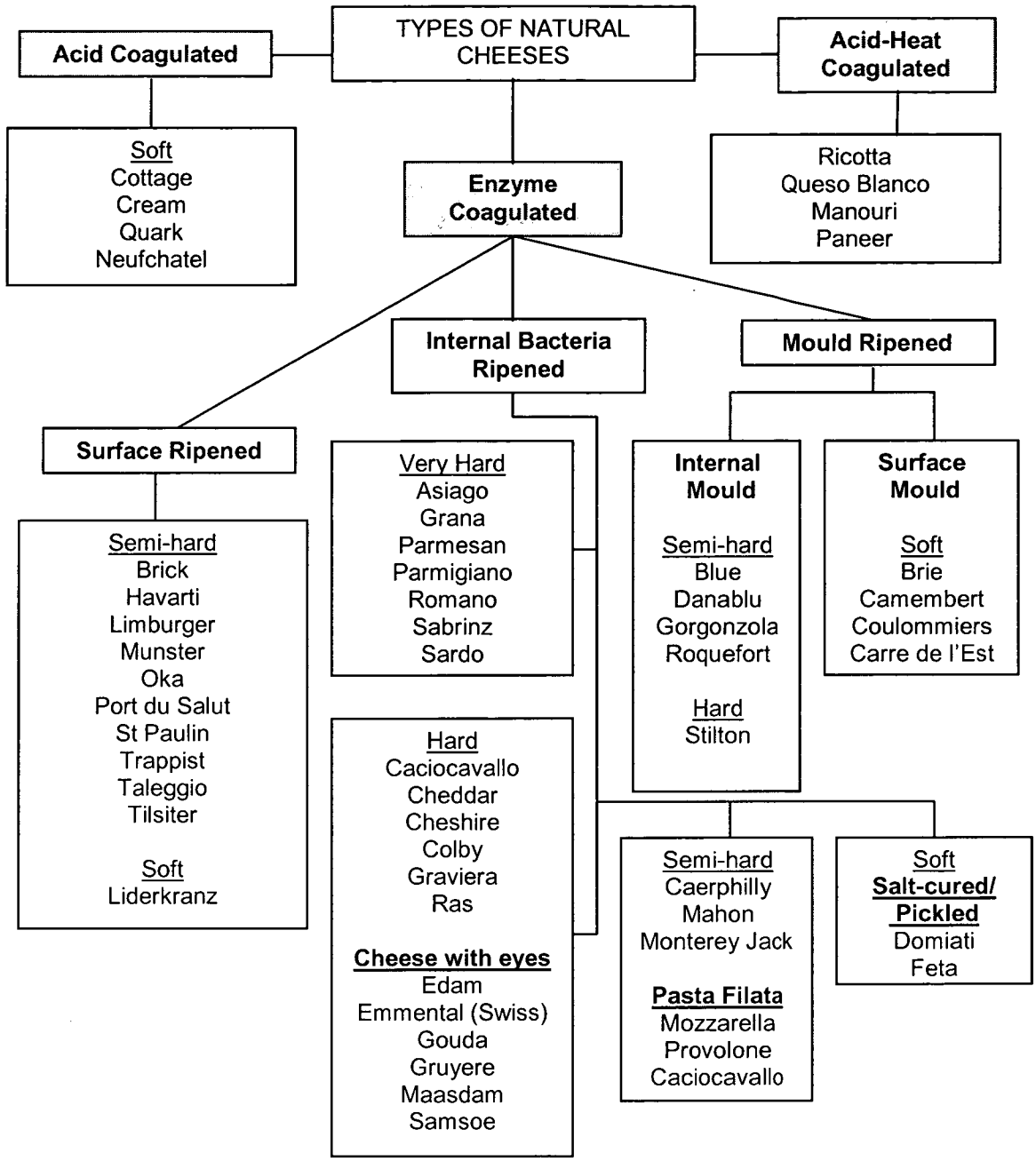


**Figure 1.1** Types of natural cheese (*After* Gunasekaran and Ak 2003).

Acid-heat coagulated cheese such as Ricotta, Queso Blanco, Paneer and Manouri are made by heating milk up to 80°C for approximately 20 minutes and then acidifying the milk by direct addition of lactic or citric acid to cause flocculation of casein and whey proteins. The combination of heat and acid causes the denaturation of whey proteins which form complexes with κ-casein and co-precipitate once the pH reaches approximately 5.5 (Farkye 2004a). These cheeses were traditionally by-products made from the whey left over from the production of rennet-coagulated cheeses but

in modern production systems, the whey is now often mixed with whole milk or the cheeses are manufactured from milk only (Johnson 1998). The curds of each cheese type are salted, pressed and consumed fresh and the presence of denatured whey protein in the curd makes these cheeses resistant to melting during cooking (Johnson 1998).

The majority of commonly consumed cheeses are made using enzymatic coagulation. The most commonly used proteolytic enzyme mixture is rennet, which is made up principally of chymosin (~95%) and some pepsin (Johnson 1998; Fox and McSweeney 2004). Rennet-coagulated cheeses make up around 75 percent of the world's cheese production and almost all ripened cheeses are part of this group. Traditionally rennet was derived from calf stomachs. In modern times, however, the calf chymosin gene has been cloned into *E. coli*, *Kluyveromyces lactis*, and *Aspergillus niger,* and the chymosin made by these organisms is used throughout the world. Other sources of proteolytic enzymes that can be used to form cheese coagulum are acid proteinases from the cardoon thistle flower, acid proteinases from certain fungi or bovine and porcine pepsins (Fox and McSweeney 2004).

Chymosin is the preferred proteinase for coagulation because it has a specific action on one peptide bond in κ-casein (Johnson 1998). The specific site of hydrolysis is the $Phe_{105}$-$Met_{106}$ bond of κ-casein, although chymosin does hydrolyse other bonds in casein when they are accessible (Johnson 1998; Fox and McSweeney 2004). When other coagulants are used, their non-specific proteolytic activity leads to excessive proteolysis that adversely affects the texture, flavour and yield of cheese. At neutral pH, casein micelles are negatively charged and have no tendency to clot due to electrostatic repulsion and stearic hindrance. The $Phe_{105}$-$Met_{106}$ bond of κ-casein is located on the *C*-terminal hydrophilic region of the casein micelle, the part that protrudes into the aqueous phase and causes stearic repulsion of the micelles. The action of the coagulant removes the hydrophilic arm and reduces the charge at the micelle surface so that the micelles are able to clot together (Johnson 1998).

After renneting, and once the desired firmness of the coagulum have been achieved, the curd is cut into cubes. The timing of this step and the size of the curd pieces are dependent upon the type of cheese being made. The smaller the size of the curd

pieces, the lower the moisture content of the cheese because the extent of syneresis is proportional to the surface are of the curd. The curd continues to shrink and expel whey after it is cut. At this point, acidification is still occurring and the rate of acidification enhances syneresis. The rate of acid development by starter cultures has a large influence over the final moisture content of a cheese. Cheese makers place a great deal of emphasis on choosing the correct starter in order to control the rate of acidification and produce cheese with desirable characteristics unique to the cheese type (Walstra et al. 1999). The cut curds are usually allowed to sit for a few minutes before the cooking process begins, where the temperature of the curds and whey mixture is increased and the curd pieces continuously stirred for a period to promote syneresis.

The time taken to cook the curds is dictated by the type of cheese being produced. Once cooking has ended, the curd is separated from the whey. Starter and adjunct cultures used in cheese manufacture are either mesophilic or thermophilic with temperature optima ~28 - 30°C or 42°C respectively (Limsowtin et al. 1996). Thermophilic starters are used for most Swiss and Italian cheeses that use a high temperature cook step as part of their manufacturing protocol. Curds of these cheeses are cooked for up to one hour at temperatures between 48 and 52°C. Thermophilic cultures are able to withstand these temperatures and then start producing acid again once the curds are placed into moulds and start to cool (Cogan and Beresford 2002). Cheeses made with mesophilic cultures use cooking temperatures up to 38°C. The most common method of whey removal, after the cook is complete, is to pour the curds and whey mixture into porous moulds that allow the whey to drain away and the curds to mat together into the shape of the mould. The shape and size of the cheese moulds is also dependent on the variety of cheese. The curd may undergo salting before or after being shaped in the moulds. The unripened cheese may be pressed in the mould to remove whey trapped in the spaces between the small curd blocks, however, not all cheeses require pressing and this step is dependent on the desired final moisture content of the cheese variety. The young unripened cheese is then ready for consumption or ripening (Johnson 1998).

### 1.1.2 The role of salt

Salting is an important step in the manufacture of cheese, it acts as a preservative, and it directly contributes to the flavour of cheese with the amount of salt used dependent on the cheese variety. Salt also influences the removal of whey from the curd, thus reducing the moisture or water activity ($a_w$) of the cheese. The reduction of $a_w$ (which is also affected by curd syneresis and ripening time) also influences microbial metabolic activity and growth, and biochemical ripening reactions. The range of salt contents of cheese varies with the variety, with acid-curd cheeses (i.e. cottage) having 0.5-0.7 per cent (w/w) and up to 4-6 per cent (w/w) in brined varieties such as Domiati and Feta (Guinee and Fox 2004). In terms of $a_w$ levels, cheeses range between 0.917 for extra hard grating cheeses such as Parmesan, and 0.97-0.988 for soft cheeses such as Brie and Cottage (Cogan and Beresford 2002). Table 1.1 lists the $a_w$ of various cheese varieties and Table 1.2 lists the minimum $a_w$ required for growth of various microbes in food. The $a_w$ of the majority of cheeses alone is insufficient to prevent the growth of yeasts and moulds and many pathogenic bacteria (Guinee and Fox 2004). It should be noted, however, that changes in $a_w$ continue throughout the ripening period of all cheeses and large $a_w$ gradients can occur between the rind (low $a_w$) and the centre of a cheese (higher $a_w$) in cheeses with long maturation times (Cogan and Beresford 2002). Vivier et al (1994) showed that the surface-growing micrococci that are partly responsible for the specific aroma of Roquefort cheese are able to grow in 20% NaCl (0.88 $a_w$) making them very well suited to the low $a_w$ which characterises the outer rind of this cheese.

**Table 1.1.** Water activity ($a_w$) of some cheese varieties (Guinee and Fox 2004)

| $a_w$ | *Cheese* |
|---|---|
| 1.00 | Fresh cheese curd, Ricotta |
| 0.99 | Beaumont, Cottage, Fresh, Quark |
| 0.98 | Belle des Champs, Münster, Pyrénées, Taleggio, Processed |
| 0.97 | Brie, Camembert, Emmental, Fontina, Limburger, Saint Paulin, Serra da Estrêla |
| 0.96 | Appenzeller, Chaumes, Edam, Fontal, Havarti, Mimolette, Norvegia, Samsø, Tilsit |
| 0.95 | Bleu de Bresse, Cheddar, Gorgonzola, Gouda, Gruyère, Manchego |
| 0.94 | Idiazábal, Majorero, Mozzarella, Norzola, Raclette, Romano, Sbrinz, Stilton |
| 0.93 | Danablu, Edelpilzkäse, Normanna, Torta del Casar |
| 0.92 | Castellano, Parmesan, Roncal, Zamorano, |
| 0.91 | Provolone, Roquefort |
| 0.90 | Cabrales, Gamalost, Gudbrandsdalsost, Primost |

**Table 1.2.** Minimum water activity ($a_w$) for microbial growth in foods (Guinee and Fox 2004)

| Pathogen | Minimum $a_w$ |
|---|---|
| Shigella spp. | 0.96 |
| *Yersinia enterocolitica* | 0.96 |
| *Vibrio parahaemolyticus* | 0.94 |
| Pseudomonas spp. | 0.95 |
| *Escherichia coli* | 0.95 |
| *Clostridium botulinum* | 0.94 |
| Salmonella spp. | 0.94 |
| *Listeria monocytogenes* | 0.92 |
| Micrococcus spp. | 0.87 |
| *Staphylococcus aureus* (aerobic) | 0.86 |
| Most yeasts and moulds | 0.80 |
| Osmophillic yeasts and moulds | 0.55 |

Cheese is salted after the curd has formed. Only one variety of cheese, Egyptian Domiati, has 5-15% NaCl added to the milk before coagulation occurs to inhibit microbial growth and maintain milk quality. The addition of salt before coagulation impairs coagulation and syneresis but this is offset in Domiati by the use of buffalo milk, which has higher casein content than cow's milk, or by the addition of skim milk powder or the addition of calcium chloride (Guinee 2004). All other cheeses are salted after coagulation using one of three methods:

- Dry salting with salt crystals added to broken or milled curd pieces (e.g. Cheddar or cottage);
- Surface dry salting where salt is rubbed onto the moulded curd (e.g. Blue cheeses);
- brine salted, where moulded curds are immersed in brine (e.g. Edam, Gouda, Provolone)
- or a mixture of all three (Fox and Cogan 2004).

Some cheeses, mainly from the Mediterranean and Balkan regions (e.g. Feta, Domiati, Haloumi), are not only brine-salted but remain in brine throughout their ripening period and are referred to as 'pickled' cheeses (McSweeney et al. 2004).

Salt plays an important role in regulating cheese microflora. Initially salt regulates the activity of starter cultures, thereby regulating the development of cheese pH, and affecting ripening, and texture (Schroeder et al. 1988). The pH is also affected by the buffering capacity of the curd and in some varieties by the washing of the curd to remove residual lactose (e.g. Dutch cheeses). Using salt to regulate the final pH of cheese is common to the British types of dry-salted cheeses such as Cheddar, Cheshire and Stilton (Guinee and Fox 2004). When these cheese varieties are hooped, the curd still has a high pH (~6.0) and acid continues to develop during the pressing step. These varieties are dry-salted at the end of hooping when pH is approaching the desired value. Fermentation of residual lactose continues in the early stages of ripening but the extent of fermentation depends on the salt tolerance of the starter, the salt-in-moisture phase (SMP) level in the curd, how quickly the salt diffuses through the curd and the extent of the salt gradient from the surface to the centre of the curd (Guinee and Fox 2004).

Residual coagulant catalyses the initial proteolysis in cheese and in hard and semi-hard cheeses $\alpha_{s1}$-casein undergoes proteolysis but β-casein does not, until much later in the ripening process (Guinee and Fox 2004). The hydrolysis of $\alpha_{s1}$-casein by rennet is increased by increasing NaCl concentrations up to 6 per cent w/w but activity of the coagulant is inhibited at higher concentrations with limited proteolysis occurring up to 20 per cent NaCl w/w (Guinee and Fox 2004). The breakdown of β-casein is significantly less than that of $\alpha_{s1}$-casein in most cheeses and its resistance to breakdown may be due to the hydrophobic nature of this casein, which is thought to make its chymosin susceptible bonds inaccessible to the action of rennet (Guinee and Fox 2004).

Together with the pH and the calcium level, salt affects the amount of *para*-casein hydration/solubility, which influences the water-binding capacity of the casein matrix, its tendency for syneresis, and its textural and cooking properties (Pastorino et al. 2003; Guinee 2004). Salt free and low-salt cheeses generally have a weak soft, elastic and pasty texture whereas highly salted varieties are harder, drier and crumblier as seen in aged cheeses and those that are brined (e.g. aged Cheddar and Feta) (Guinee and Fox 2004). Cheeses with greater moisture contents/higher $a_w$ are softer in texture and have better melt ability but have poor grating characteristics as

seen in Mozzarella and other pasta filata melting cheeses (Gunasekaran and Ak 2003).

### 1.1.3 Ripening

Cheeses that are not consumed fresh undergo a controlled ripening (maturation or curing) period that can last anywhere from two weeks (e.g. Mozzarella) to greater than 2 years (e.g. Vintage Cheddar, Parmesan). The period of ripening is generally inversely related to the final moisture content of the cheese (Fox and McSweeney 1997). Some cheese varieties can be consumed at different stages of ripening with cheese matured the longest often referred to as 'mature' or 'vintage'. The ripening of a cheese allows the natural process of microbiological and biochemical changes that began in the first stages of manufacture to develop flavours and textures unique to the individual variety of cheese. Biochemical changes occur during ripening due to the catalytic action of the coagulant, indigenous milk enzymes, starter culture enzymes, secondary microflora, and exogenous enzymes added by cheese makers (Fox et al. 2000). Physical parameters are also manipulated to influence ripening and include primarily, the storage time, temperature and humidity (Scott et al. 1998).

During ripening protein, fat, lactose, lactate and citrate are hydrolysed to varying extents (Farkye 2004b). These primary reactions are followed by secondary catabolic changes such as amino acid and fatty acid catabolism and related reactions, and the catabolism of lactic acid to carbon dioxide and water (Fox and McSweeney 2004). Residual rennet together with natural proteases and those from starter cultures or adventitious bacteria cause further proteolysis of α- and β-casein into large peptides which are then broken down to smaller peptides and amino acids which are soluble and directly affect cheese flavour and mouth feel/texture (Gunasekaran and Ak 2003; Farkye 2004b). In mature Cheddar for instance, one third of the milk protein has been catabolised to forms that are soluble at pH 4.6 (Gunasekaran and Ak 2003). Further catabolism of amino acids releases flavour compounds unique to each cheese, and dependent upon non-starter lactic acid bacteria (NSLAB) and other enzymes present in the curd (Law 2001).

Triglycerides in all cheeses undergo hydrolysis by indigenous, endogenous and/or exogenous lipases (McSweeney 2004b). Lipolysis releases significant flavour compounds essential to the final smell and taste of certain cheeses (hard Italian, Feta, mould-ripened, Blue vein and goat cheeses). Lipolysis is limited in other varieties (Swiss and semi-hard cheeses) where high levels induce flavour defects such as rancidity (Beuvier and Buchin 2004). Lipolytic agents in cheese originate from the milk (lipoprotein lipase or LPL), the coagulant (only rennet pastes) and microflora (LAB and adjunct microbes) (McSweeney 2004b). LPL is an important lipase in raw milk cheeses as it is largely inactivated by pasteurisation. Commercial rennet extracts are free from lipase, however rennet paste, made directly from macerating young dairy animal stomachs, is used as the coagulant in the manufacture of the Italian cheeses such as Provolone and Pecorino and also in Greek Feta (McSweeney 2004b). *Penicillium camemberti* produces lipases responsible for the extensive lipolysis that characterises Blue cheeses, and lipases produced from it and the Gram-positive surface microflora found on smear cheeses contribute to lipolysis in the white mould-ripened varieties such as Brie (McSweeney 2004b).

The pH of a cheese is determined by acidification during manufacture and the buffering capacity of the curd (McSweeney 2004b). Cheese texture is directly related to the pH due to the effect of pH on solubility of caseins (McSweeney 2004b). In general, high pH cheeses have softer curds than low pH cheeses (e.g. high pH Camembert compared to low pH Cheddar). Metabolism of lactose *via* glycolysis is almost complete in the early stages of ripening, and up to 90 per cent of the residual lactose is lost in the whey. The retained fraction is rapidly metabolised to L-lactate after whey drainage (Cogan and Beresford 2002; McSweeney 2004a). In the early stage of ripening, the rate of glycolysis is determined by the temperature and the salt-in-moisture phase (SMP) levels in the curd (McSweeney 2004b). In some cheese varieties (i.e. Cheddar and other dry-salted cheeses), SMP levels rapidly increase and starter activity ceases, while unfermented lactose is then metabolised by NSLAB. When populations of NSLAB are high and/or levels of lactose remain high, the residual lactose is fermented into large quantities of D-lactate or through racemisation of L-lactate to DL-lactate (McSweeney 2004b). Racemisation favours the formation of white Ca-DL-lactate crystals in the cheese curd that, while harmless, often cause consumer rejection of cheese on the basis that the cheese is mouldy or

contaminated with foreign particles and is a problem mainly in Cheddar and Dutch-type cheeses (McSweeney 2004a; 2004b). Lactate is an important substrate for a range of reactions that have both positive and negative influences on cheese ripening. LAB can oxidise lactate to acetate, ethanol, formate and carbon dioxide depending on how much oxygen is available. In brine-salted cheeses *Clostridium tyrobutyricum* metabolises lactate to butyrate and $H_2$ causing the late gas blowing defect, consisting of cracks in the curd and off flavours, and is caused when NaCl levels have not adequately diffused through the curd to reach levels inhibitory to *C. tyrobutyricum* (McSweeney 2004b). The metabolism of lactate is most important in Swiss and surface mould-ripened cheeses. *Propionibacterium freudenreichii,* which gains access to the milk from the environment or is purposely added as an adjunct culture, metabolises lactate to propionate, acetate, $CO_2$ and $H_2O$. Propionate and acetate contribute to the distinct flavour of Swiss cheese and $CO_2$ migrates in the curd until it reaches a weak spot where it accumulates and forms the 'eyes' common to Swiss cheese (McSweeney 2004a). *P. camemberti* on the surface of Camembert and Brie cheeses, metabolise lactate at the surface causing an increase of the surface pH, which leads to the development of a pH-gradient, and the migration of lactate to the surface (McSweeney 2004a). High surface pH also causes precipitation of calcium phosphate leading to further migration of calcium and phosphate to the surface. These changes cause the characteristic soft liquid-like texture of these cheeses. Smear ripened cheeses (i.e. Tilst and Limburger) have yeasts on their surface which metabolise lactate to cause a decrease in the surface pH and encourages the growth of Gram-positive bacteria on the surface (McSweeney 2004a).

During ripening the $a_w$ is further reduced due to evaporation of water from the cheese surface and this is an important part of the maturation of cheeses subject to long ripening periods, such as the Italian extra hard grating cheeses or vintage Cheddar, with their characteristic crumbly dry texture and unique acidic (low pH) and salty (high NaCl) flavours. Excess moisture loss is controlled using 80 - 90 per cent relative humidity levels in ripening rooms or by dipping cheese wheels in wax or plastic, since loss of too much water adversely affects the final texture of the cheese. $A_w$ is also significantly reduced by the extensive hydrolysis of proteins and to a lesser degree by hydrolysis of lipids. For each peptide or ester bond hydrolysed, one molecule of water is consumed (Cogan and Beresford 2002).

The temperature used for ripening cheese is one that promotes ripening reactions and the growth of secondary cultures but also minimises the growth and proliferation of spoilage and pathogenic microbes. Cheddar cheese is usually ripened at 6-8 °C and mould and smear ripened cheeses are ripened at between 10-15°C. Some cheeses, such as Emmental and Roquefort, go through several time-temperature combinations during their ripening period and those temperatures usually vary between 10 and 22°C (Cogan and Beresford 2002). Accelerated ripening is promoted at higher temperatures but this also causes adverse changes to the texture and flavour.

The process of cheese manufacture and ripening involves a number of complex microbiological and biochemical events, which develop the diversity of flavour and texture characteristics of the vast variety of cheeses available. The preceding discussion gives a brief overview of the basic processes that occur during cheese manufacture. Extensive reviews can be found in Cheese Chemistry, Physics and Microbiology Volumes 1 and 2 edited by Fox et al (2004) together with publication from Gunasekaran and Ak (2003) and Law (2001).

### 1.2 Raw Milk Cheese

Approximately 700 000 tonnes of raw milk cheese (about 10 per cent of total cheese production in the European Union) is produced in Europe annually with the largest contributions from France, Italy and Switzerland (Grappin and Beuvier 1997). The majority of these cheeses are considered to be artisanal and represent years of tradition in specific territories and some traditional European raw milk cheeses have protected designation of origin (PDO) status (Bertozzi and Panari 1994). Many segments of the community prefer cheeses made from raw milk and regard such cheeses as being better tasting and more nutritious than those made from heat treated or pasteurised milk. Advocates believe that exceptional tasting cheeses can only be made with raw milk and those made with heat-treated milk have a dull and subdued character (Rampling 1996).

Traditionally artisanal raw milk cheeses were made on farm from the milk of the farms' own herds and sold to the local community. Cheese flavour and texture would vary throughout production due to seasonal changes in animal feed and

condition and the changing nature of the indigenous microflora found in the raw milk. However, modern cheese-manufacturing technologies have had to evolve with increasing consumer demand and larger markets, and in modern production of raw cheeses, there is a tendency to consolidate the milk from smaller producers into larger lots for most cheese varieties (Leedom 2006). This has caused changes not only in milk production methods, but also in milk collection methods with milk collected over wider areas and mixed and increased transport and storage times with consequences for milk quality (Beuvier and Buchin 2004). The mixing of milk from different sources and longer storage times induces the development of microbial communities in the raw milk which differ to those present in milk used from single farms, increasing the need for improvement of on-farm hygiene to produce milk with lower microbiological counts. In Europe this is enforced by European Microbiological Standards (Beuvier and Buchin 2004). Raw milk of good microbiological quality (low counts) together with the use of milk standardisation and specific starter cultures and secondary adjunct cultures has led to raw milk cheeses being produced commercially with more constant and uniform quality than ever before (Beuvier and Buchin 2004).

Cheese made from raw milk has a diverse and rich microflora not found in cheese made from pasteurised or other heat-treated milk. Organisms found in raw milk come from the animal's udder and hide and from milking utensils (Jay 2000; Leedom 2006). Raw milk may have present bacteria from some or all of the following genera: *Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Lactobacillus, Microbacterium, Oerskovia, Propionibacterium, Micrococcus, Proteus, Pseudomonas, Bacillus*, and *Listeria* and at least one coliform genus (Jay 2000). Some of these bacteria contribute to the unique flavour and texture of many raw milk cheeses, others, however, cause spoilage or are pathogenic. Milk taken aseptically from a healthy cow's udder contains typically lower than $10^2$-$10^3$ cfu/mL. Heavily contaminated teats, however, have been shown to contribute up to $10^5$ cfu/mL with similar microbial counts in milk from udders infected with mastitis (Adams and Moss 2000). This indicates the importance of good animal hygiene and health practices and adequate cleaning of milk handling equipment, for the production of raw milk cheeses.

The production and consumption of raw milk cheeses is widespread in Europe and is part of the culture of dining in many nations such as France and Italy. On the other side of the world Canada permits the importation and production of raw milk cheese, provided the cheese has been stored at a temperature of 2°C or more for a period of 60 days or more. In addition, cheese made from an unpasteurised source must not contain more than 500 *E. coli* or 1,000 *S. aureus* per gram (Anonymous 2008). United States regulations require cheese to be pasteurised or, as an alternative treatment, cheeses made from raw milk must meet the minimum 60-day aging period requirement. The 60-day aging requirement is currently being reviewed for soft and semi-soft cheeses in Canada and the United States (Caro and Garcia-Armesto 2007; Civera et al. 2007; D'Amico et al. 2008). However, interstate trade of raw milk products within the US is prohibited (Anonymous 2008).

In Australia, a number of European raw milk cheeses are currently imported. They have been assessed on the principle of equivalence whereby the technologies and methods used to manufacture the raw cheese are deemed to produce a cheese that has an equivalent level of safety as the same product made from pasteurised milk. In 1998, a number of Swiss cheeses were approved for sale in Australia. The raw cheeses Emmental, Gruyere and Sbrinz were deemed to meet an appropriate level of safety due to the use of high curd cooking temperatures during manufacture, and the cheeses Appenzellar, Tilsiter and Vacherin were produced using thermised milk, and so already complied in part with Australian food regulations. In Australia thermised products are required to be made from milk heated to no less than 62°C for 15 seconds or more and then storing cheese for more than 90 days before sale (Anonymous 2008). The cheese Tête de Moine was not permitted because a microbiological safety assessment could not confirm the manufacturing process would provide an equivalent level of safety to cheese made in accordance with Australian regulations in force at that time (Anonymous 2008). In 2002 Italian extra hard grating cheeses made from raw milk were approved for sale because the manufacture of extra hard grating cheeses meets the equivalence requirement and thus the cheeses are considered not to not pose any significantly greater public health and safety risk. The final cheese must, however, contain less than 36 per cent moisture, be matured for greater than 6 months, and be prepared using a curd cooking temperature of at least 48°C (Anonymous 2005). In 2005, the French blue-

mould ripened cheese, Roquefort, was also approved for sale based on the equivalence principle. All imported raw cheeses must comply with the microbiological limits for food set out in The Australian Food Standard 1.6.1 (FSANZ 2009). These limits and the sampling plans specified in the Standard for *E. coli* in all cheese and *L. monocytogenes* in raw milk cheese is shown in Table 1.3.

**Table 1.3.** The sampling plans specified in Standard 1.6.1 for Escherichia coli for all cheeses and for Listeria monocytogenes in all raw milk cheese (Anonymous 2005).

| Food | Microorganism | n | c | m | M |
|---|---|---|---|---|---|
| All cheese | *Escherichia coli* | 5 | 1 | 10 | $10^2$ |
| All raw milk cheese (cheese made from milk not pasteurised or thermised) | *Listeria monocytogenes*/25g | 5 | 0 | 0 | 0 |

n = the minimum number of sample units which must be examined from a lot of food

c = the maximum allowable number of defective sample units (the number of samples they may exceed 'm')

m = the acceptable microbiological level in a sample unit.

M = the level which, when exceeded in one or more samples, would cause the lot to be rejected.

The imported raw milk cheeses available for sale in Australia are hard cheeses that have a curd cooking step (for the Italian and Swiss varieties only), and have low pH values with low $a_w$ and high salt levels, conditions that may be unsuitable for *L. monocytogenes* and *E. coli*, with their numbers tending to reduce during maturation (Anonymous 2008). In Tasmania, pasteurisation has been compulsory for all cheese makers since 1993 prior to which manufacturers producing less than 30 tonnes of cheese per year were permitted to make raw milk cheese (Willman 1998). In 2008, cheese maker Nick Haddow of Bruny Island Cheese Tasmania, obtained permission from FSANZ to produce raw hard cheese with the same restrictions as those for Italian raw extra hard grating cheeses (Reeves 2009).

Worldwide, both raw and pasteurised dairy products, especially cheeses, have a good safety record (Alterkruse et al. 1998). A review of epidemiological literature for the period 1948 to 1988 by Johnson et al (1990a; 1990b; 1990c) found six outbreaks of illness were due to cheese produced in the USA during that period. The use of raw milk was a factor in only one outbreak and the rest were most frequently caused by

post-pasteurisation contamination. Milk and milk products were implicated in 5 per cent of the bacterial outbreaks reported in France between 1988 and 1997, and in 1 to 5 percent of the reported outbreaks from the USA, Finland, Netherlands, England, Wales, Germany and Poland for the period 1983 to 1997 (De Buyser et al. 2001). For each country, raw and pasteurised products were equally involved (30 per cent each) in outbreaks. This statistic may, however, obscure the fact that more pasteurised milk cheese may be produced, so that proportionally, pasteurised milk cheese is less involved per unit of production. The other 40 per cent of outbreaks were caused by products where the milk treatment regime was unspecified. A recent review of the control of hygiene factors in the production of raw milk cheese by Donnelly (2001) found no reason to consider aged raw milk cheeses any less safe than those made from heat-treated milk.

The growth and survival of pathogens in raw cheese is dependent on the cheese variety and the manufacturing technology used (Grappin and Beuvier 1997). Pathogens grow more readily in soft cheeses with high moisture content, high pH (>5.5) and high $a_w$ than in hard, dry, long-ripened cheeses with low pH (Ryser and Marth 1987b; Genigeorgis et al. 1991; Back et al. 1993; Eppert et al. 1997; De Buyser et al. 2001; Leedom 2006; Meunier-Goddik et al. 2008). Of the soft high-risk varieties, surface-ripened cheeses, such as Camembert, Brie and the smear cheeses, represent the greatest risk for the outgrowth of pathogens (Johnson et al. 1990a). Smear cheeses such as Limburger, Brick, and Tilsiter, have high ripening temperatures of between 8 and 19°C (dependent on the variety) over several months to promote the development of the microbial progressions that produce the characteristic smear on the cheese surface. The organisms in the smear also cause the pH of the surface to increase from pH 5.0 to 7.5 during ripening, making the surface an area with conditions enabling the growth of pathogens (Cogan and Beresford 2002). Several studies have shown that surface ripened cheeses can support the growth of pathogenic organisms. Maher and Murphy (2000) showed that the rind of two smear-ripened farmhouse raw milk cheeses provided favourable conditions for the growth of *E. coli* and *S. aureus*. Little and Knøchel (1994) demonstrated that growth of the psychotropic pathogen Yersinia enterocolitica could occur over a temperature range 4 to 20°C on Brie, while Salmonella and Bacillus cereus grew only at 20°C. During an investigation designed to simulate post

manufacture contamination, Genigeorgis et al (1991) showed that Brie and Camembert supported the growth of *L. monocytogenes* over the temperature range 4 – 30°C. This was attributed to the high pH at the surface (7.4) and the centre (6.9) of the samples. Starter cultures, present at levels of up to $10^8$ cfu/g did not appear to have any adverse effect on growth of *L. monocytogenes* in Brie or Camembert (Genigeorgis et al. 1991). Ryser and Marth (1987b) inoculated *L. monocytogenes* onto the surface of Camembert cheese, stored at 6°C, and observed 2- to 3- $\log_{10}$ increases in three out of four strains after 70 days of storage. Back et al (1993) observed growth of *L. monocytogenes* in Camembert, Brie and Ricotta manufactured from inoculated milk. Growth was also observed when Camembert cheese was surface-inoculated and stored at 3 and 6°C. D'Amico et al (2008) found that when introduced as a post processing contaminant, *L. monocytogenes* grew on soft surface mould ripened cheeses (Camembert-type) stored for more than 60 days.

An *L. monocytogenes* surveillance study by Loncarevic et al (1995) of 333 cheeses produced or imported into Sweden, found 6 percent of the samples contaminated with *L. moncytogenes*. Of the positive samples, the pathogen was more frequently isolated in soft and semi-soft cheeses made from raw milk (42 per cent) than in those made from pasteurised milk (2 percent). However, a study of European red smear cheeses in retail outlets by Rudolf and Scherer (2001) found a higher incidence of *L. monocytogenes* in pasteurised smear cheeses (8.0 per cent) than in those made from raw milk (4.8 per cent). The presence of pathogens in cheeses made from heat-treated milk is most likely the result of post-pasteurisation contamination as demonstrated in one example by Canillac and Mourey (1993)where contamination by *L. monocytogenes* was found to have been caused by the washing of surface ripened cheeses with contaminated brushing machines.

Contamination of Vacherin Mont d'Or soft cheese with *L. monocytogenes* led to an outbreak of human Listeriosis in Switzerland during the 1980s. In this case the pathogen was isolated from the cellars where the cheese was matured and the wooden benches and brushes used during ripening (Bula et al. 1995). The first documented outbreak of illness caused by *E. coli* occurred in 1971 when surface ripened soft French cheeses (Camembert, Brie and Coulommiers) sold in the United states were implicated (Bell and Kyriakides 1998). It was not reported whether the

implicated cheeses were made from raw or pasteurised milk, however, French authorities found *E. coli* O124 in the tanks used to coagulate the milk. In the 40 years between 1948 and 1988 six outbreaks of food borne illness in the USA were caused by cheese and the most frequent contributing factor was reported to be post-pasteurisation contamination (Johnson et al. 1990a). Several types of soft surface-ripened cheeses, such as Brie, were more involved in those outbreaks than harder cheeses such as Cheddar. Extra hard Italian grating cheeses were not responsible for any reported illnesses (Johnson et al. 1990a).

### 1.2.1 The behaviour of *L. monocytogenes* and *E. coli* in Raw Milk Cheese

*L. monocytogenes* and *E. coli* are considered high-risk pathogens to the Australian cheese industry (Sandman 2008). Both are contaminants of raw milk and have been responsible for numerous recalls and a few high profile outbreaks caused by the consumption of both raw and pasteurised cheeses.

*L. monocytogenes* can enter raw milk from environmental or faecal contamination or from being shed in the udder of an animal suffering from, sometimes asymptomatic, mastitis, encephalitis or *Listeria*-related abortion (Ryser 1999; Leedom 2006). The pathogen can grow in the presence of up to 10 per cent NaCl concentrations, which corresponds to an aw of 0.92, and in the pH ranges 4.5 to 9.6 (Cole et al. 1990; Pearson and Marth 1990; Ross et al. 2000). *L. monocytogenes* also has the ability to respond to sub-lethal stresses by inducing mechanisms to protect the cell from subsequent lethal stresses (Hill and Gahan 2000). The acid tolerance response (ATR) is one adaptive response that is important in cheese making, where exposure to a mild non-lethal pH allows cells to subsequently endure exposure to lethal pH for longer, with enhanced survival shown to be at least 1000-fold (Hill and Gahan 2000). *L. monocytogenes* also has psychrotrophic properties enabling it to grow at refrigeration temperatures, however the lag phase increases with decreasing temperature and the maximum doubling time is 1-2 days at 4°C (Pearson and Marth 1990; McLauchlin 2006). The temperature range for growth of *L. monocytogenes* is 1-45°C with the optimum between 30-37°C (Pitt et al. 1999).

In humans *Listeria* infection can cause listeriosis which has a serious invasive form that may cause foetal infections and abortion, meningitis, and encephalitis (Hill and Gahan 2000). For most healthy individuals listeriosis produces very mild influenza-like symptoms or no symptoms at all, however, serious complications occur in individuals who are immuno-compromised, pregnant, elderly or the very young with a fatality rate between 30 and 40 per cent, although epidemiological investigations show that the incidence of infection is rare (Hill and Gahan 2000; Midelet-Bourdin et al. 2006).

In 1983, an outbreak of listeriosis in Massachusetts, USA, was epidemiologically linked with the consumption of pasteurised milk (Ryser 1999). In 1985, an outbreak of listeriosis was caused by the consumption of contaminated soft Mexican-style cheese in California, where pasteurised milk had been mixed with raw milk. These well-publicised outbreaks prompted a number of surveys worldwide to determine the extent of *L. monocytogenes* contamination in raw milk (Ryser 1999). The pathogen has been shown to occur at low levels in 3.2, 2.3 and 3.6 per cent of raw bovine milk samples in the United States, Canada and Europe respectively (Ryser 1999). This percentage of incidence was calculated by pooling data from a number of published surveys, where different methodologies were used and different seasonal fluctuations were apparent. As such, the incidence may be underestimated. Overall, the prevalence of *L. monocytogenes* in bulk milk is sporadic with seasonal variation and when present, levels are typically below 3 CFU/mL (Lovett et al. 1987; Meyer-Broseta et al. 2003).

Sutherland and Porritt (1995) surveyed twelve cheese factories in eastern Australia, for the presence of *L. moncytogenes*. The overall incidence was 12 per cent with individual factories ranging from 0-35 per cent, results that are similar to overseas studies (Sutherland and Porritt 1995). A study of the incidence of *L. monocytogenes* on equipment and environmental sites in dairy processing plants by Pritchard et al (1995) found positive equipment (non-product contact surfaces of machinery and equipment) isolates in 28.6 per cent of the factories tested and positive environmental (floors, drains and walls) isolates in 90.5 percent of plants. The potential threat of post processing contamination depends on how well *L. monocytogenes* persists in the factory environment, and environmental

contamination does not always translate into contamination of equipment in the same factory. *L. moncytogenes*, however, is known to persist in factories and form biofilms attached to surfaces in food-processing plants, increasing its resistance to antimicrobial agents (Arnold 1998; Beresford and Andrew 2001). It is clear from such surveys that the incidence of *L. monocytogenes* in raw milk and in factories is problematic for producers of cheese as it represents a high risk of contamination and because it can grow during the shelf life.

*E. coli* live commensally in the gastrointestinal tract of most mammals without causing disease (Karns et al. 2007). Most strains of *E. coli* are non-pathogenic, however, the emergence over the last twenty years of pathogenic strains with and low infective doses and significant morbidity and mortality has raised concerns within the cheese making industry. Pathogenic *E. coli* are grouped according to their O:H serotypes, clinical syndromes, virulence factors and pathogenicity mechanisms (Chung et al. 2006). The most important in terms of the severity of disease, are the enterohemorrhagic (EHEC) strains, such as *E. coli* O157:H7, which can cause haemolytic colitis including the complications haemolytic uraemic syndrome and thrombocytopenia purpura, which are potentially fatal in children, the elderly and the immunocompromised (Bell and Kyriakides 1998; Katic and Radekov 1998; Karns et al. 2007). This strain is also characterised having a low infective dose, as little as 50 cells may cause illness (Tilden et al. 1996). As with *L. moncytogenes*, *E. coli* can enter raw milk from faecal contamination or shedding from an infected udder (Bell and Kyriakides 1998). Since the EHEC *E. coli* strain O157:H7 emerged as a food borne pathogen in 1982, after an outbreak caused by consumption of undercooked ground beef in the United States, it (and other EHEC strains) have caused outbreaks associated with the consumption of contaminated pasteurised and raw milk (Ryser 2001). Various EHEC *E. coli* have been shown to occur in between 0.75 to 3.8 percent of the bulk milk samples in the United States and Canada (Karns et al. 2007). The percentage of occurrence of *E. coli* in bulk milk is dependent upon the strain tested since some surveys report occurrences for particular EHEC strains (such as *E. coli* O157:H7) only and not occurrence levels for other pathogenic strains.

The minimum growth temperature for *E. coli* O157:H7 has been reported as approximately 8°C (Buchanan and Bagi 1994; Bell and Kyriakides 1998) and the

optimum and maximum temperatures are approximately 37 and 46°C respectively (Salter et al. 1998). The temperature ranges for growth of *E. coli* also vary with strain. *E. coli* will not grow at refrigeration temperatures (3-7°C), however, pathogenic strains have been shown to survive for several weeks with 0.5 to 1.5 $\log_{10}$ reductions in populations (Bell and Kyriakides 1998). *E. coli* are able to grow between the pH range of 3.9 to 9.0 (Presser et al. 1997; Bell and Kyriakides 1998), however outbreaks of food borne illness caused *E. coli* O157:H7 have demonstrated its significant acid tolerance in foods such as apple cider (pH 3.6-4.0) (Besser et al. 1993), yoghurt (pH 4.5-4.6) (Morgan et al. 1993), and mayonnaise (pH 3.65-4.44) (Weagant et al. 1994). The minimum $a_w$ for growth of *E. coli* is 0.95, however *E. coli* O157:H7 is known to survive the fermentation and drying processes used to make fermented meat products with low $a_w$ (0.90) and low pH (4.5-4.8) (Bell and Kyriakides 1998).

A number of challenge studies have been undertaken to investigate the behaviour of *L. monocytogenes* and *E. coli* in a number of artificially contaminated laboratory made cheeses. Most research on pathogen survival and inactivation during cheese making uses milk with high initial inoculum levels in order to assess the extent of inactivation adequately, however, Dalgaard and Jørgensen (1998) suggest naturally contaminated products should be used for challenge tests, as this is more reflective of contamination in real situations. They argue that pathogens in naturally contaminated milk that survive fermentation and/or heat treatment are probably relatively few in number and not in the same physiological state as inoculum cultured under favourable conditions. Their work showed that *L. monocytogenes* growth in naturally contaminated cold-smoked salmon was much slower than growth in inoculated challenge tests (Dalgaard and Jørgensen 1998). The use of naturally contaminated milk to investigate pathogen behaviour during cheese making in order to achieve results directly relevant to conditions found in the factory is commendable but this may not always be feasible since contamination rates of raw milk are erratic at best as shown in surveys of *L. monocytogenes* contamination levels (Ryser 1999). Milk artificially inoculated to a high level allows for quantitative evaluation of the kinetics of inactivation and allows researchers some control over their ability to achieve data relevant to a food, which is chemically and in many cases microbiologically, highly complex. A number of challenge studies investigating the

behaviour of *E. coli* and *L. monocytogenes* in various cheeses are reviewed below. Direct comparison between studies is not always easy, however, mainly due to the fact that different bacterial strains are often used and some studies report values of pH and $a_w$ while other report results as titratable acidity, lactic acid concentration and percentage salt.

Many challenge studies on *E. coli* and *L. monocytogenes* survival in fermented dairy products have been conducted in soft cheeses. Concern about the safety of soft cheeses made from both raw and pasteurised milk abounds due to the number of outbreaks attributed to them as discussed in Section 1.2. Soft cheeses are characterised by high moisture contents, high $a_w$ and generally high pH which allow pathogens to survive and, sometimes, to grow. Maher et al (2001) manufactured smear ripened cheese with milk inoculated with *E. coli* O157:H7. Cheeses were stored at 15°C for 18 days for smear development and then at 6°C for the remainder of ripening. The pathogen was inoculated at an initial level of 1.52 $\log_{10}$ cfu/ml and grew to 3.4 $\log_{10}$ cfu/ml after the first day. Numbers decreased to less than 10 cfu/ml after 21 days but the pathogen was still detected after 90 days with enrichment (Maher et al. 2001). The pH of the smear ripened cheese rind was approximately 5.27 but reached 6.32 after 10 weeks due to the growth of smear micro flora. The interior of the cheese was initially pH 4.89 and reached 5.16 after 10 weeks. Back et al (1993) investigated the behaviour of *L. monocytogenes* in Camembert cheese made with inoculated milk, and in a range of commercial cheeses surface inoculated after manufacture. *L. monocytogenes* was inoculated at a level of approximately 3 x $10^2$ cfu/ml for laboratory-made Camembert and cheeses stored at 3, 6, 10 and 15°C. Un-inoculated pieces of laboratory-made Camembert were also dipped into suspensions of *L. monocytogenes* containing around $10^4$ cfu/ml and cheese pieces were stored at 6, 10 or 15°C. Finally a range of commercially produced soft and semi-soft cheeses were surface inoculated and stored at 3, 5-6 and 10°C (Maher et al. 2001). In inoculated Camembert *L. monocytogenes* grew at the cheese surface with a 100 fold increase after 40 days observed at 3 and 6°C and 3-4 $\log_{10}$ increases at the higher temperatures. Growth was only observed in the cheese centres at 15°C. In Camembert pieces dip-inoculated growth was observed at all temperatures in both the centre and at the surface of the cheese (Maher et al. 2001). The authors observed little or no growth of *L. monocytogenes* on blue or white mould Stilton cheese, no

growth on blue and white mould Lymeswold cheese, Brie, Chaume, soft cheese with garlic and herbs or Mycella cheese, however, the organism survived and viable cells were still present at each sampling. Ryser and Marth (1989) made Brick cheese (a Limburger-type smear ripened cheese) from pasteurised milk containing $1x10^2$cfu/ml of *L. monocytogenes* and ripened at 15°C and then later at 10°C.

*L. monocytogenes* strains Scott A and Ohio grew rapidly in Brick cheese even during smear development but strains V7 and California failed to grow during smear development (Ryser and Marth 1989). Strains V7 and California were still detected in cheeses after 22 weeks but strains Scott A and Ohio decreased 1- to 7- fold during weeks 20 - 22. Morgan et al (2001) manufactured soft lactic cheeses using raw goat milk inoculated with $10^2$ cfu/ml of *L. moncytogenes*. Bacterial entrapment in the curd cause a 7- fold increase in cell numbers followed by a decrease until day 14 where numbers stabilised. Viable cells were still detectable after 42 days by enrichment (Morgan et al. 2001). Data from this study was used in the meta-analysis described in Chapter 2 of this thesis. *L. monocytogenes* survived for more than 120 days in soft Turkish Kashar cheese even after heat treatment of the ripe curd at 75°C for 5 minutes (Çetínkaya and Soyutemí 2004). *L. monocytogenes* numbers decreased substantially but the pathogen was able to survive for more than 90 days at 4°C during the manufacture and ripening of soft Turkish White cheese (Erkmen 2001). (That study was not included in the Chapter 2 meta-analysis due to insufficient data available for calculation of inactivation rates).

Challenge studies conducted to assess the survival of *E. coli* O157:H7 during the manufacture of Cheddar cheese, show an increase of over one $\log_{10}$ cfu/g during fermentation. This is likely to have been caused by concentration of cells within the curd during the coagulation, although limited growth may contribute to the increase (Spahr and Url 1994; Reitsma and Henning 1996; Schlesser et al. 2006). Reitsma and Henning (1996) inoculated *E. coli* into pasteurised milk at high ($1 \times 10^3$ cfu/mL) and low (1 cfu/mL) levels from which Cheddar cheese was made. Cheeses were dry-salted, ripened at 6 – 7°C and reached a final pH between 4.95 and 5.2. During cheese ripening, levels of *E. coli* in the high inoculum cheese decreased by 2-$\log_{10}$ cfu/mL after 60 days, but *E. coli* were still present after 158 days. Cheeses made with low inoculum levels had *E. coli* cells present after 130 days of ripening but no

cells were detected at day 158. The mean salt-in-moisture phase (SMP) of these cheeses was 3.25 per cent yet Donnelly (2001) states that average SMP levels in normal Cheddar should be between 5 and 5.5 per cent. Reitsma and Henning (1996) acknowledge that the low SMP may have affected their results and recommended studies in Cheddar with higher SMP levels. Schlesser et al (2006) inoculated raw milk with *E. coli* O157:H7 from which they made Cheddar cheese which was ripened at 7°C. They also observed a 1- to 2- $\log_{10}$ increase in *E. coli* cell numbers during fermentation and pressing, with numbers then decreasing over the ripening period. After 60 days the initial cell numbers had decreased by one $\log_{10}$ but cells were present in three out of five replicates after 158 days of maturation. In another study by Ryser and Marth (1987a) three strains of *L. monocytogenes* (Scott A, V7 or California) were inoculated at a level of 500 cells per ml of pasteurised milk which was used to make Cheddar cheese that was ripened at 6 or 13°C with a final pH of 5.0 to 5.1. An increase of 0.1 to 0.8 $\log_{10}$ cfu/g during fermentation and pressing was also observed in that study, with greatest numbers of *L. monocytogenes* ($5 \times 10^3$ cfu/g) detected after 14 days of ripening. *L. monocytogenes* numbers decreased during further ripening but strains California, Scott A and V7 were still present after 154, 224 and 434 days of ripening respectively (Ryser and Marth 1987a). Data from that study were included in the meta-analysis in Chapter 2. In each challenge study Cheddar was made to standard manufacturing specifications, with normal ripening periods, and the results indicate that the pH, salt levels, $a_w$ and maturation period are not adequate to ensure the inactivation of *L. monocytogenes* or *E. coli* contaminants in milk used for cheese making. From the results in those studies, it is evident that *L. monocytogenes* and *E. coli* are able to survive beyond the minimum 60-day aging requirement required in USA for raw cheeses, and is further as shown in the other studies as described below.

Raw Gouda cheese, a hard cheese similar to Cheddar which was aged for a minimum of 60 days, was associated with an outbreak of hemorrhagic colitis in Canada in 2002, caused by *E. coli* O157:H7 (Honish et al. 2005). The outbreak was the first confirmed *E. coli* O157:H7 infection associated with raw hard cheese in Canada and the pathogen was present in the cheese still enclosed in factory sealed packaging 104 days after production, indicating that the ageing process was inadequate to eliminate enteric pathogens (Honish et al. 2005). The time required for pathogen inactivation

also depends on the load of pathogens initially present and the presence of *E. coli* in raw Gouda fully 104 days after manufacture may also have been reflective of the initial pathogen load and not just the ageing process.

Bachmann and Spahr (1995) investigated the survival of *E. coli* and *L. monocytogenes* in Swiss hard (Emmentaler) and semi-hard (Tilsiter) raw-milk cheeses. The hard cheese was cooked at 53°C for 45 minutes and the semi-hard cheese was cooked at 42°C for 15 minutes as per the standard manufacturing process for these varieties. Both cheeses were brine-salted and ripened at 11-13°C for 90 days. The final pH of the cheeses was 5.5-5.8. Neither pathogen could be detected after the first day of ripening in the hard cheese with inactivation presumed to have been caused by the high curd cook temperatures used. *E. coli* was not detectable after 60 days of ripening in the semi-hard cheese, however, *L. monocytogenes* was still detectable after the 90 day ripening period. Curd cook temperatures in the semi-hard cheese were considered by the authors to not be high enough to cause inactivation of pathogens. Data for the semi-hard cheese in this study were included in the meta-analysis in Chapter 2. Dominguez et al (1987) investigated the behaviour of *L. monocytogenes* during the manufacture of a Manchego-style semi-hard cheese made from a mixture of sheep, goat and cows milk (15:35:50 ratio) with final pH between 5.1 and 5.8. Pasteurised milk was inoculated with either $1.9 \times 10^5$ or $4 \times 10^3$ cells per ml. Small increases in the number of cells present was observed during the early stages of manufacture and cell numbers at both inoculum levels decreased during the ripening period but were still present after 60 days of ripening. Similarly Yousef and Marth (1988) manufactured the semi-hard cheese Colby (average pH 5.0), using pasteurised milk inoculated with *L. monocytogenes* at between 100 and 1000 cells per ml, with cheeses ripened for 140 days at 4°C. Cell numbers increased by over one $\log_{10}$ during fermentation and remained constant during the initial stages of ripening before decreasing steadily during maturation. Cells were still present after 140 days in some cheeses with the rate of inactivation dependent upon the strain used, the moisture content of the cheese and initial cell concentrations (Yousef and Marth 1988). Cheeses made with higher initial inoculum levels persisted longest as did cheeses inoculated with the strain V7. A few cheese batches were produced with moisture levels greater than those legally allowed in Colby cheese (i.e., >40%) which, according to the author, facilitated longer survival

times for *L. monocytogenes* due to their moisture levels (Yousef and Marth 1988). Data from this study were included in the meta-analysis in Chapter 2.

Hicks and Lund (1991) inoculated three batches of cottage cheese with *L. monocytogenes* which were then stored for 14 days at 4, 8 and 12°C. *L. monocytogenes* numbers decreased throughout the 14 days of storage, and inactivation was greatest in batches of cottage cheese with the lowest initial pH and highest initial lactic acid concentration (Hicks and Lund 1991). Piccinin and Shelef (1995) inoculated ten 'store bought' cottage cheese with *L. monocytogenes* and then stored them at 5, 10 and 20°C for 35 days. Five cheeses were manufactured in USA and five in Canada. The mean pH of purchased cheese was 5.05 for USA cottage cheeses (remaining stable during storage) and 4.89 for Canadian cottage cheeses (decreasing to 4.77 after 24 days at 5°C). The mean inoculum level for the USA cheeses was 3.27 $\log_{10}$ cfu/g and the mean for Canadian cheeses was 3.90 $\log_{10}$ cfu/g. *L. monocytogenes* numbers declined over the course of the experiment but the organism was still detectable at day 35 at both pH levels and each temperature (Piccinin and Shelef 1995). The duration of the experiment in that study was one which exceeded the recommended shelf life of the 'store-bought' cheeses. Inoculum levels could be considered higher than what may be expected in naturally contaminated cheeses and this is common when undertaking challenge studies in foods. However, the initial pathogen load in the cottage cheeses may have extended the duration of their survival since the time taken for pathogen inactivation is related to the initial pathogen load. That cottage cheese did not support the growth of *L. monocytogenes* is clear but the pathogen may not have been detected at day 35 had the initial inoculum levels been lower.

Papageorgiou and Marth (1989a) investigated the behaviour of *L. monocytogenes* during the manufacture and ripening of Blue cheese. Cheese was made using pasteurised milk inoculated with 1.0 – 2.0 x $10^3$ *L. monocytogenes* of both strains Scott A and California and ripened at 9 – 12°C for 84 days before being stored at 4°C. Prior to hooping population numbers in the curd were, on average, one $\log_{10}$ greater than those in the inoculated milk, presumably due to cell entrapment in the curd. Numbers in cheese increased, however, by a further 0.58 – 1.22 $\log_{10}$ (dependent upon the strain) during the first 24 hours of manufacture and the authors

concluded that this increase was due to growth of *L. monocytogenes* (Papageorgiou and Marth 1989a). Once the pH of cheese decreased to 5.0 and below (after approximately 24 hours) all growth of *L. monocytogenes* ceased. Populations of the pathogen decreased rapidly during the first 50 days of ripening after which (between day 50 and 120) growth of the mould *P. roqueforti* caused the pH to increase resulting in survival (i.e. cessation of inactivation), but not growth, of *L. monocytogenes* (Papageorgiou and Marth 1989a). Strain Scott A survived without change in numbers through to day 120 while strain California was only detectable after day 80 to 120 through cold enrichment (Papageorgiou and Marth 1989a). Data from (Papageorgiou and Marth 1989a) was not used in the meta-analysis in Chapter 2 because a ripening temperature range (9 – 12°C) was provided instead of a single temperature.

Papageorgiou and Marth (1989b)made Feta from pasteurised cows milk inoculated with 5.0 x $10^3$ *L. monocytogenes* (Scott A or California strain). Fresh cheese was placed into 12% brine for 24 hours at 22°C, and then placed into 6% brine for 4 days at 22°C and then 4°C for the remainder of the ripening period. *L. monocytogenes* increased by 1.5 $\log_{10}$ cfu/g during the first 2 days of ripening until the pH was reduced to 4.6 after which growth ceased (Papageorgiou and Marth 1989b). While no further growth occurred after the first two days, cells of both strains were still present after 90 days of ripening despite the low pH of 4.30 (Papageorgiou and Marth 1989b). Govaris et al (2002b) investigated the behaviour of *E. coli* O157:H7 in Feta and Telemes cheeses made from ewe's and cow's milk respectively. Milk was inoculated with 5.1 $\log_{10}$ cfu/mL of *E. coli* and both cheeses were made with either thermophilic (T) or mesophilic (M) LAB starter cultures. The pathogen increased in numbers in both cheeses during the first 24 hours of production before decreasing in all trials. The increase in cell density is attributable to entrapment of cells in the curd but may also be partly due to a small amount of growth although it is difficult to establish whether that was the case as increases were between 0.82 and 1.56 $\log_{10}$ cfu/g. Fresh cheeses were dry salted and held at 16°C until the pH was reduced to 4.6 when they were then stored in brine at 4°C for the remaining ripening time (Govaris et al. 2002b). *E. coli* numbers continued to decrease during ripening and were not detectable in Feta cheese after 44 (T) and 36 (M) days and in Telemes cheese after 40 (T) and 30 (M) days respectively. Govaris et al (Govaris et al.) also

investigated the survival of *E. coli* O157:H7 in Feta and its brine after 2 months of ripening when the pathogen was introduced as a brine contaminant rather than a milk one. Feta was made using ewe and goats' milk free of *E. coli* O157:H7 and approximately 11kg of cheese was stored in 2.5kg of brine at either 4 or 12°C. The brine was inoculated with *E. coli* with final distribution approximately 5.3 $\log_{10}$ cfu/mL) of brine (Govaris et al. 2002a). Populations of *E. coli* in the brine decreased over the time of the experiment, however, numbers in Feta increased to a peak at approximately 4 days before declining at a similar rate to those in brine. The pathogen was still detectable, via cold enrichment, in the brine after 44 and 36 days at 4 and 12°C respectively and in Feta after 44 and 38 days respectively (Govaris et al. 2002a). The pH of Feta and brine reached 4.44 at 4°C and pH 4.0 at 12°C and *E. coli* survival was longer at 4°C (Govaris et al. 2002a). Hudson et al (1997) investigated the survival of bioluminescent *E. coli* O157:H7 in Cottage, Colby, Romano and Feta cheeses made from pasteurised milk inoculated with *E. coli*. The authors observed a 3-$\log_{10}$ reduction in the population of *E. coli* O157:H7 after 27, 30 and 27 days for Colby, Romano and Feta cheeses respectively. The pathogen did not survive the cooking temperature of 56°C in Cottage cheese.

Spano et al (2003) made Mozzarella cheese using raw milk inoculated with *E. coli* O157:H7 to a level of $10^5$ cfu/mL. Mozzarella cheese undergoes a high heat treatment during a process of curd stretching which enhances its melt ability. Directly after curd stretching the cheese is traditionally brine salted in approximately 23 per cent brine for 12 hours (Spano et al. 2003). In this work two different heating temperatures (70 and 80°C) were used to stretch the Mozzarella curd and the viability of *E. coli* determined for both. Stretching in hot water at 80°C for 5 minutes was more effective in inactivating *E. coli* than stretching in water at 70°C. No viable cells were detected immediately after stretching at 80°C. Stretching at 70°C did not immediately inactivate *E. coli* but a marked reduction in numbers was seen after brining and cells were not detected after storage at 4°C for 7 days (Spano et al. 2003). The authors provide no reason for the survival of *E. coli* after stretching in hot water at 70°C. It is surprising that both stretching temperatures did not inactivate *E. coli* in the same manner since they are well above the uppermost limit for the growth of the majority of vegetative bacteria, however, as noted below Buazzi et al (1992b) stretched Mozzarella curd in water at 77°C for 3-4 minutes and

the curd only reached 65°C for two minutes. Stretching the curd at 70°C may have resulted in the actual temperature experienced by *E. coli* in the curd being less than 60°C which may have allowed the survival of a small proportion of cells. That the salting in brine caused a marked decrease in the numbers of *E. coli* may have been in part due to the cells being injured during the stretching at 70°C. In work by Buazzi et al (1992b) Mozzarella was made using pasteurised milk inoculated to contain $10^4$ to $10^5$ CFU *L. monocytogenes* per ml. Numbers of *L. monocytogenes* in freshly cut curd were 25 to 38 per cent greater than in inoculated milk. Cooking curd at 40°C for 30 minutes caused a decrease of approximately 38 per cent compared to numbers after cutting and numbers during cheddaring of curd increased by 25 per cent, over numbers at the end of cooking. Temperature alone could not have caused the inactivation of *L. monocytogenes* since lethal temperatures for this pathogen are greater than 45°C (Pitt et al. 1999). During cheese manufacture the pH of the curd reduces during the cook step (see Figure 3.2) but cheese pH usually takes at least 6 – 8 hours to reach levels that may be inhibitory for pathogens so this factor also does not explain the inactivation seen by (Buazzi et al. 1992b) and in fact the authors state that at the end of the cook period the pH was 5.90. Cheese curd was stretched for 3 – 4 minutes in water at 77°C which caused complete inactivation of *L. moncytogenes*. During stretching the curd reached 65°C and remained there for two minutes (Buazzi et al. 1992b). Kim et al (1998) found stretching time and temperature were very important for controlling *L. moncytogenes*. In this study different heating times and temperatures for curd stretching were investigated for Mozzarella made from pasteurised milk inoculated with either 7 or 3 $\log_{10}$ CFU per gram *L. moncytogenes*. No starter was used and cheeses were instead acidified with citric acid. Curd stretching was undertaken at 55, 66 and 77°C for 1, 3, and 5 minutes (Kim et al. 1998). Stretching at 55°C had a minimal effect on *L. monocytogenes* numbers, stretching at 66°C reduced numbers by 5 logs and stretching at 77°C for 1 minute inactivated *L. monocytogenes* to below detectable levels in Mozzarella cheese. Where stretching temperature partially reduced numbers it was found that brining for 12 hours at 4°C had a lethal effect on populations of *L. monocytogenes* that remained (Kim et al. 1998). In the studies by Spano et al (2003) and Kim et al (1998) it appears that after damage from heated stretching, brining was more lethal. However, no information is provided in those studies as to whether a control cheese, that did not receive a heat treatment, showed better pathogen survival after brining. It could

be possible that the inactivation was due only to the brine, and not to the combination of heat and the brine but this conclusion cannot be made from the data in these studies.

Northolt et al (1988) investigated the behaviour of *L. monocytogenes* during the making of Dutch cheeses. Gouda and Maasdam cheeses were made from pasteurised milk inoculated with 5.0 x $10^2$ cfu/ml of *L. moncytogenes*. Fresh cheeses with pH 5.5 were brine salted at 13°C after which Gouda cheeses were ripened at 13°C for 6 weeks. Maasdam cheeses were ripened for 2 weeks at 13°C, then 2 weeks at 18°C and thereafter at 4°C (Northolt et al. 1988). Numbers of *L. monocytogenes* were concentrated in the curd by a factor of ten during the formation of the curd and limited growth occurred (an increase of a further factor of four) and numbers remained constant during the 6 week ripening period (Northolt et al. 1988). The final pH after ripening was not reported.

Giannou et al (2009) investigated the behaviour of *L. monocytogenes* in Greek Graviera cheeses. Graviera cheeses are made as large whole cheeses weighing up to 15 kilograms which are then portioned at the point of sale and either sold directly or wrapped in vacuum bags and refrigerated until sold (Giannou et al. 2009). Pieces of fully ripened Graviera cheese (> 90 days old) were surface inoculated with *L monocytogenes* to yield approximately $10^3$ cfu/$cm^2$ of cheese then packaged under vacuum, or air, and stored at either 4, 12, or 25°C. The typical pH of these cheeses is between 5.2 and 5.6 and $a_w$ is 0.94 – 0.96. *L. monocytogenes* did not grow on Graviera cheese but the authors note it was slowly inactivated, in each treatment (Giannou et al. 2009). After 30 days viable counts of *L. monocytogenes* on cheese stored at 25°C, especially under vacuum packaging, were significantly lower than those stored at 4 or 12°C. After 90 days survival was highest in samples stored at 4°C in vacuum packaging, with less than one $\log_{10}$ reduction observed. Data from this study were not included in the meta analysis in Chapter 2 because inadequate data was available to determine rates of inactivation.

Yousef and Marth (1990) examined the survival of *L. monocytogenes* in Parmesan cheese. Cheese milk was inoculated with $10^4$ – $10^5$ cfu/mL and an increase in cell numbers by 0.61 to 1.0 $\log_{10}$ was observed during the curd cooking step and was

attributed to entrapment of cells by the curd. The curd was cooked for 45 minutes at 51°C and pressed curds were brine salted for 7 days before being dried for 4 to 6 weeks. Cheeses were then vacuum packed for the remainder of the 10 month ripening period. At the end of the ripening period cheese pH was between 5 and 5.1 and moisture levels were between 30.1 and 31.4 per cent. Rapid, almost linear, decline in the populations of *L. monocytogenes* in Parmesan cheese was reported in this study from the time of press until between 2 and 16 weeks of ripening (depending on pathogen strain and cheese batch) after which it was not detected in any samples. Rapid inactivation of *L. monocytogenes* in Parmesan was attributed to the decrease in $a_w$ that occurred during the ripening period with syneresis enhanced by the high curd cooking temperature, the higher ripening temperature used (12.8°C) and the addition of lipase for flavour (Yousef and Marth 1990).

Panari et al (2004) investigated the behaviour of *L. monocytogenes* and *E. coli* during the manufacture of Parmigiano-Reggiano (Parmesan) cheese. Cheese curd was cooked for 12 minutes at 56°C and held at 55°C for at least one hour before moulding. Cheeses were brine salted for 20 to 30 days and ripened at between 18 and 20°C for 10 to 12 months (Panari et al. 2004). No cells of either species were detected after the first 24 hours of manufacture, nor were they detected at any stage in subsequent brining and ripening steps. The authors attribute the rapid inactivation of *L. monocytogenes* and *E. coli* to the temperature of the cook and the rapid development of thermophilic LAB as the curds cool which produced a very rapid drop in pH to 5.0 during the first few hours of processing (Panari et al. 2004). Inactivation data for *L. monocytogenes* from Yousef and Marth (1990) and Panari et al (2004) were included in the meta-analysis in Chapter 2.

Pellegrino and Resmini (2001) investigated the safety of Italian Grana cheeses (Grana Padano and Parmigiano Reggiano) and concluded that several parameters associated with the manufacture of extra hard grating cheeses contribute to their microbiological safety. Those parameters include cooking and holding the curd for up to 85 minutes at temperatures of 53 to 56°C, moulding the cheese while still hot thus keeping the curd at over 50°C for up to 10 hours, brine salting which helps to reduce the $a_w$ to approximately 0.90, and ripening periods of between 9 and 12

months which further reduce the $a_w$ to levels inhibitory to the survival of pathogens (Pellegrino and Resmini 2001).

Many of the challenge studies presented here used pasteurised milk inoculated with pathogens rather than naturally contaminated or artificially inoculated raw milk. Raw milk contains natural inhibitors and a complex array of natural biota, the removal of which (through pasteurisation) could create an artificially protective environment for pathogens (Pellegrino and Donnelly 2004). Even if raw milk used for cheese production receives a mild heat treatment (sub-pasteurisation) (Pellegrino and Donnelly 2004), the environment experienced by pathogens in the milk may still be vastly different to that in pasteurised milk in terms of the complexity of the synergistic effects of natural flora and inhibitors together with the addition of starters and salting of the curd by cheese makers. Using raw milk for challenge studies would allow more realistic conclusions to be made about the safety of raw milk cheese especially in light of the current move in Australia for cheese makers to petition FSANZ for permission to make and sell raw milk cheese in this country.

### 1.3 The Acid Tolerance Response in *E. coli* and *L. monocytogenes*

*L. monocytogenes* is a hardy bacterium known for its ability to survive in many harsh environments. It has been shown to survive the manufacturing and ripening processes of many low pH foods such as cheese (see Section 1.2.1), orange juice, salad dressing and other fermented dairy products such as yogurt where it was found for nearly 30 days after manufacture and where the pH was as low as 4.0 (Choi et al. 1988; Farber and Peterkin 1991; Gahan et al. 1996). When exposed to adverse environments, such as low pH in foods, gastric secretions, or the phagocytes of a host cell, *L. monocytogenes* invokes mechanisms to allow it to adapt and survive. It has been conclusively shown that exposure to a sub-lethal pH (5.0 to 6.0) induces an acid-tolerance response (ATR) in *L. monocytogenes* enhancing its survival when it is later exposed to a lethal acid shock (pH 3.5 to 4.0) (Kroll and Patchett 1992; Gahan et al. 1996; O'Driscoll et al. 1996; Ferreira et al. 2003; Koutsoumanis et al. 2003). Cataldo et al (2007) investigated the survival of acid adapted and non adapted *L. monocytogenes* in soft Italian cheeses and found no difference in the growth and survival rates except in Crescenza cheese which showed significantly increased

growth of acid-adapted *L. monocytogenes* cells compared with Mozzarella, Gorgonzola and Ricotta cheeses. Non-adapted cells recovered from Mozzarella and Crescenza exhibited high tolerance to lethal pH also (Cataldo et al. 2007). Gahan et al (1996) showed that acid adapted *L. monocytogenes* cells were able to survive for longer periods in acidified dairy products and during active milk fermentation than non adapted cells. This effect was also shown in *Salmonella typhimurium* by Leyer and Johnson (1992) where cells exposed to sub-lethal levels of HCL (pH of 5.8) had increased resistance to inactivation by organic acids commonly present in cheese (lactic, propionic and acetic), and acid-adapted cells also showed enhanced survival during milk fermentation and in various cheeses stored for two months at 5°C. Similarly acid-adapted *E. coli* O157:H7 showed increased resistance to lactic acid, survived better than non-adapted cells during sausage fermentation and showed enhanced survival in apple cider (pH 3.4) and shredded salami (pH 5.0) (Leyer et al. 1995).

Initiation of the ATR has also been shown to provide cross protection to other stresses. O'Driscoll et al (1996) showed that adaptation of *L. monocytogenes* to mildly acidic conditions for one hour provided cross protection against osmotic shock and Lou and Yousef (1996) demonstrated adaptation to acid significantly increased the resistance of *L. monocytogenes* to heat. Induction of an ATR causes regulation of the synthesis of a number of proteins, including various virulence factors (Sokolovic et al. 1993; Phan-Thanh and Gormon 1995). The pathogenicity of *L. monocytogenes* is associated with its acid resistance. O'Driscoll et al (1996) and Conte et al (Conte et al. 2000; Conte et al. 2002) have demonstrated that induction of the ATR is linked to virulence of *L. monocytogenes* and the ATR is considered a *sine qua non* for successful murine infection (Marron et al. 1997).

The ability of pathogens to initiate an ATR is profoundly relevant to cheese production systems because the microbiological safety of cheese is initially dependent upon acid formation by LAB during fermentation. Cheese manufacture requires the rapid development of acid during fermentation for the development of flavour, texture, to prevent the growth of spoilage organisms and for microbiological safety (Gahan et al. 1996). If fermentation is slowed or does not adequately proceed pathogens present in the milk could have an opportunity to initiate an ATR which

would influence their survival in an otherwise lethal food environment. A detailed discussion of the protein systems *L. monocytogenes* employs when it inducts an ATR can be found in Section 5.4.

EHEC *E. coli* strains have been shown to survive in acidic foods such as unpasteurised apple cider (apple juice) (Besser et al. 1993; Zhao et al. 1993), yogurt (Morgan et al. 1993; Massa et al. 1997), and mayonnaise and mayonnaise based sauces and dressings (Weagant et al. 1994). Leyer et al (1995) showed that prior exposure of *E. coli* O157:H7 cells to mildly acidic pH increased the strain's resistance to lactic acid, fermentation during sausage making and in fermented apple cider when compared with non-adapted cells. Work by Lin et al (1996) on the same strain of *E. coli* characterised several genetic aspects of acid tolerance and identified that several acid resistance systems potentially contribute to the survival of *E. coli* in acidic food and within the GI tract. In the work undertaken in this thesis, both *E. coli* and *L. monocytogenes* were used in challenge studies in cheese. During the manufacture of cheese the potential for pathogens to become acid resistant is a real threat. The Food Safety Centre Group at the University of Tasmania has been working extensively on characterising the genetic and proteomic responses of *L. monocytogenes* to survival in adverse environments. It was, therefore, decided that the final piece of work in this thesis would be to characterise the proteomic response of *L. monocytogenes* during cheese fermentation to expand the group's data set.

### 1.4 The non-thermal inactivation of *L. monocytogenes* and *E. coli* in cheese

It is well known that reducing pH and water activity to levels below that which permit the growth of bacteria will preserve food. While it is well known that severe pH and/or $a_w$ preclude growth, the role of non-thermal temperature in the growth inhibition and inactivation of *L. monocytogenes* and *E. coli* in such inimical environments is less well described. As the focus of the current study, this chapter now turns to a more comprehensive review of this topic.

Fermentation of raw materials has long been used to extend shelf life and to improve the safety of a number of foods. Fermented meat products are often produced from

raw meat with no bactericidal heat treatment and pathogens are inhibited due to the combination of pH, reduced $a_w$, lactic acid and curing salts (nitrate and/or nitrite) and spices (McQuestin et al. 2009). However, a number of outbreaks of infection caused by *E. coli* have been epidemiologically linked to uncooked fermented meat products (Williams et al. 2000; Schimmer et al. 2008) leading investigators to look at the kinetics of inactivation of *E. coli* during the production of fermented meats (Clavero and Beuchat 1996; Hinkens et al. 1996; Calicioglu et al. 1997; Faith et al. 1998; Cosansu and Ayhan 2000; Chikthimmah and Knabel 2001; Calicioglu et al. 2002). A preliminary study by Ross and Shadbolt (2001) produced a predictive mathematical model to assess the lethality of fermented meat processes for *E. coli* and performed a meta-analysis of 60 inactivation rates for temperatures in the range of 4 – 50°C, temperatures which of themselves are not lethal for *E. coli*. Results of the analysis suggested that *E. coli* inactivation under growth-preventing conditions of pH, $a_w$ and other factors (inimical conditions) was strongly influenced by temperature and was described by an Arrhenius model (Ross et al. 2008). From a data set with varied pH (3.5 – 7), $a_w$ (0.75 – 0.95) and temperature (4 – 50°C), numerous strains, product formulations and processes, temperature was found to explain 66 % of the variance in ln-transformed inactivation rate data (Ross et al. 2008). McQuestin et al (2009) conducted a more expansive collation and analysis of the relative influence of pH, $a_w$ and temperature upon the survival of *E. coli* in fermented meats and analogous broth systems. The data used by McQuestin et al (2009) for their meta-analysis included pH in the range 2.8 – 6.14, $a_w$ between 0.75 – 0.986 and temperatures in the range -20 – 66°C. A wide variety of strains and product formulations were included. The collated inactivation data was presented as an Arrhenius plot (*ln* inactivation rate *vs.* 1/absolute temperature) and showed that temperature accounted for 61 per cent of the variance in the transformed data while pH and $a_w$ accounted for only 8 per cent of the variability (McQuestin et al. 2009). The results of these meta-analyses revealed the dominance of non-lethal temperatures on the inactivation of *E. coli* in fermented meats and led to the identification of manufacturing recommendations that, based on these results, would improve product safety. Those recommendations include increasing the temperature and time of manufacture whilst ensuring that pH and $a_w$ reach inimical levels (McQuestin et al. 2009). Ross et al (2008) speculated that, under inimical conditions, non-lethal temperature is the key factor influencing the rate of

inactivation of all vegetative bacteria, regardless of species, when they are prevented from growth by other factors such as pH and $a_w$. Zhang et al (2010) investigated whether the same inactivation mechanisms might apply to the gram-positive bacterium *L. moncytogenes.* Those authors showed that the rate of inactivation of *L. monocytogenes* is also strongly influenced by temperature when other factors limit growth. They also compared rates of non-thermal inactivation of *E. coli* and *L. monocytogenes* in broth at pH 3.5 and $a_w$ 0.90 and at temperatures in the range 5 to 45°C and found no systematic differences in inactivation rates of those species except at 45°C, at which *L. monocytogenes* exhibited biphasic inactivation kinetics and rapid initial inactivation rates compared with *E. coli.* Arrhenius analysis of data sets for the inactivation of various strains *L. monocytogenes* and *E. coli* indicated that temperature accounted for 91 and 94 % respectively of the variation in the transformed data. Their results were consistent with the hypothesis that the effect of non-lethal temperature on the rates of inactivation is species independent.

The increasing rate of bacterial inactivation with increasing temperature within the non-thermal range is in agreement with several studies of *E. coli* and *L. monocytogenes* inactivation in different food types and analogous broth systems. Goepfert et al (1968) showed inactivation of *S. typhimurium* was greater at 13°C than at 7.5°. Cole et al (1990) found that low pH and high salt concentration inactivated *L. monocytogenes* in a temperature dependent manner and concluded that low temperatures (5 and 10°C) allow *L. monocytogenes* to survive low pH and high salt concentration for longer than at 30°C because metabolism and growth are reduced at low temperatures (Cole et al. 1990). Tienungoon et al (2000) investigated the minimum $a_w$ required for growth of *L. monocytogenes* and found that the minimum $a_w$ value for growth increased as incubation temperature decreased. This observation was also made by Farber and Peterkin (1991). Giannou et al (2009) found that survival of *L. monocytogenes* in Greek Graviera cheeses stored at 4, 12 or 25°C was greatest at 4°C. Overall, in their experiment they found that lower storage temperatures facilitated slower inactivation rates for *L. monocytogenes* (Giannou et al. 2009). Results of the study by Govaris et al (2002a) for *E. coli* in Feta cheese indicate the pathogen survived for longer in Feta and brine at 4°C than at 12°C.

In conclusion, cheese is a widely consumed ready-to-eat food that has the potential to cause food borne illness. Pathogens such as *E. coli* and *L. monocytogenes* have been associated with outbreaks of illness linked to the consumption of both pasteurised and raw fermented dairy products. The challenge studies reviewed above indicate that both pathogens have the potential to survive in all but the very hardest and driest of cheeses. Raw milk cheeses are viewed by many consumers as a superior product to cheeses made with pasteurised milk yet there is great potential for raw milk to harbour pathogens which may go on to be present in the final cheese product and cause illness. In Australia cheese makers are currently petitioning FSANZ to change its rulings and allow the domestic production of raw milk cheeses. It is vital, therefore, to understand the potential for survival of pathogens as a function of cheese type and processing conditions and to see whether survival is enhanced in raw versus pasteurised cheese and whether temperature has the greatest effect on inactivation. Of particular interest is the behaviour of pathogens during the fermentation step and during maturation (as this period is of concern also for post-manufacturing contamination) and whether optimisation of processing and preservation of non-heat treated foods could be achieved through the manipulation of temperature.

### 1.5 Objectives

Within the above context, the aim of this thesis is to expand understanding of the effects of temperature, low pH and $a_w$ on the kinetics, and mechanisms of inactivation of *E. coli* and *L. monocytogenes* in raw milk cheeses. This will be approached specifically by:

1) a) Following on previous studies (Ross and Shadbolt 2001; McQuestin 2006) of the importance of temperature on governing the inactivation rate of *E. coli* in fermented meat products, characterising the role of temperature on the kinetics of inactivation of *L. monocytogenes* once sufficient hurdles are in place to prevent growth. The inactivation of populations of *L. monocytogenes* exposed to low $a_w$ (0.90) and low pH (3.5) in laboratory media is analysed as a simple well-controlled laboratory system that imitates conditions relevant to fermented cheese products. b) Following on the meta-

analysis of the relative effect of temperature, pH and $a_w$ on the inactivation of *E. coli* in fermented meat by McQuestin et al (2009), published inactivation data for *L. monocytogenes* is assessed via meta-analysis in an attempt to identify the main factors that influence the inactivation of *L. monocytogenes* in cheeses and analogous foods.

2) Evaluating the effect of ripening temperature on the rate of inactivation of *E. coli* and *L. monocytogenes* in laboratory-made semi-hard cheese made from both raw and pasteurised milk. The results are analysed to reveal and quantify basic patterns of response and compare it to literature data.

3) Evaluating the effect of ripening temperature on the rate of inactivation of *E. coli* and *L. monocytogenes* in laboratory-made Roquefort-style blue cheese made from raw ewe's milk. The results are analysed to reveal and quantify basic patterns of response and compared to literature data and the responses in the semi-hard cheese studies.

4) Evaluating protein expression profiles of *L. monocytogenes* in response to lactic acid fermentation. The potential for *L. monocytogenes* to induce an ATR in cheese fermentation systems is examined by proteomic methods to ascertain the induction of stress-related proteins.

## Chapter 2 The effects of non-thermal temperature, pH and water activity on *L. monocytogenes* inactivation determined by experiments in laboratory broth systems and by meta-analysis.

### 2.1 Introduction

In the majority of foods microorganisms experience a range of stressful conditions that determine the food's suitability as a medium for survival and growth (Adams and Moss 2000). Food production systems use the deliberate application of stresses that inhibit microbial growth and survival to produce microbiologically safe and stable foods. This concept is known as the hurdle effect or hurdle concept (Leistner 2000). The hurdle concept is widely used in many food production systems that do not have a heat treatment step as a means of growth inhibition and inactivation of bacterial pathogens (Shadbolt et al. 2001). Fermentation is one such process. During fermentation $a_w$ and pH are the most important factors used to affect the growth and survival of microorganisms. Some bacteria, however, have been shown to survive the food environment produced by this type of processing for extended periods of time (Glass et al. 1992).

Pathogenic *E. coli* have recently been associated with severe food-borne illnesses arising from the consumption of fermented meat products such as salami and other cured meat products (Centers for Disease Control and Prevention 1995; Tilden et al. 1996; Williams et al. 2000; MacDonald et al. 2004) which do not have a bacteriocidal heat treatment step during production but, rather, rely on reduced pH and low $a_w$ to confer safety (Zhang et al. 2010). These outbreaks led to research into the kinetics of *E. coli* inactivation during the manufacture of fermented meats (Clavero and Beuchat 1996; Hinkens et al. 1996; Calicioglu et al. 1997; Cosansu and Ayhan 2000; Chikthimmah and Knabel 2001; Calicioglu et al. 2002). Furthermore, Ross and Shadbolt (2001) observed that, within the range of temperature, pH and $a_w$ found in fermented meat processes, rates of inactivation of *E. coli* in fermented meat products were greatly affected by temperature. Those authors found that pH and $a_w$ had less influence on the rate of inactivation of *E. coli* but rather served to produce an environment that prevented growth. Subsequently, data from Zhang et al (2010)

and McQuestin (2006), have supported the observations of Ross and Shadbolt (2001).

Specifically, McQuestin et al (2009) undertook a meta-analysis of published inactivation data for *E. coli,* under a wide range of growth-preventing pH and $a_w$ conditions, and for temperatures in the range of -20 to 66°C. Inactivation data collated from independent studies were presented as an Arrhenius plot (*ln* inactivation rate *vs.* 1/absolute temperature) and indicated that temperatures in the biological range 0 to 47°C accounted for 60% of the variance in the observed *ln* inactivation rate data for all of the data collated by McQuestin et al (2009).

Ross et al (2008) speculated that, under inimical conditions, non-lethal temperature is the key factor influencing the rate of inactivation of all vegetative bacteria, regardless of species, when they are prevented from growth by other factors such as pH and $a_w$. Zhang et al (2010) showed that rate of inactivation of the Gram-positive bacterium *L. monocytogenes* is strongly influenced by temperature when other factors limit growth. They compared rates of inactivation between *E. coli* and *L. monocytogenes* in broth at pH 3.5 and aw 0.90 and at temperatures in the range 5 to 45°C and found no systematic differences in inactivation rates between those species. Their results were consistent with the hypothesis that the effect of non-lethal temperature on the rates of inactivation is species independent.

*L. monocytogenes* is a gram-positive organism and differs from *E. coli* in both physiology and its ability to tolerate harsh environments such as high salt, low pH, low $a_w$ and very low temperatures (Ryser 1999). *L. monocytogenes* has been associated with a number of major outbreaks of listeriosis from the consumption of contaminated raw and pasteurised milk, butter and soft cheeses (Linnan et al. 1988; Bula et al. 1995; Goulet et al. 1995; Adams and Moss 2000; Lundén et al. 2004; Makino et al. 2005). A severe outbreak of listeriosis in United States in 1985 was traced to soft Mexican-style pasteurised cheese that was contaminated with raw milk (Linnan et al. 1988). There were 142 cases and 48 deaths and that outbreak marked *L. monocytogenes* as a serious emerging food borne pathogen (Ryser 1999). Cheese is a fermented dairy product with many different styles. Most of the cheeses involved in serious outbreaks of illness have been classified as soft cheeses, which

traditionally have a pH between 6-7 and high water activity with moisture contents generally greater than 40% (Linnan et al. 1988; Bula et al. 1995; Goulet et al. 1995; Fox and McSweeney 2004). However, *L. monocytogenes* has been shown to survive during the making and ripening of semi-hard cheeses such as Cheddar, Colby and Blue (Ryser and Marth 1987a; Yousef and Marth 1988; Papageorgiou and Marth 1989a). These products are similar to fermented meat products in that, during the production of semi-hard cheeses, a combination of low $a_w$, low pH and competition with starter cultures has traditionally been used to prevent the growth and survival of pathogens (Morgan et al. 2001; Lundén et al. 2004).

To better understand the kinetics of inactivation of *L. moncytogenes*, and its potential to survive in semi-hard cheese, several studies were undertaken and are reported here. In the first, a series of inactivation studies in broth adjusted to pH 3.5 and $a_w$ 0.90 were undertaken in the temperature range 5 to 45°C. A further two series of inactivations in broth at pH 4.5 and either $a_w$ 0.93 or 0.95 at 15°C were undertaken to expand the original dataset. The parameters for the second set of inactivations were chosen using a predictive model for growth limits of *L. monocytogenes* based upon Tienungoon et al (2000). Inactivation rates were plotted on Arrhenius co-ordinates and lines of best fit determined by simple linear regression. The Arrhenius model generated was compared to the Arrhenius models for inactivation of *L. monocytogenes* and *E. coli* reported elsewhere (McQuestin et al. 2009; Zhang et al. 2010).

Finally, a meta-analysis of unpublished and published inactivation data for *L. monocytogenes* was also undertaken to quantify patterns of inactivation in environments analogous to those found in semi-hard and hard cheeses. Data from a variety of published and unpublished experiments, performed in both broth systems and food products, were collated. The change in numbers of *L. monocytogenes* over time in both broth studies and published data was used to calculate inactivation rates using simple linear regression. Inactivation rates were plotted on Arrhenius co-ordinates and lines of best fit determined by simple linear regression. The Arrhenius model generated was compared to recently published models for non-thermal inactivation of *E. coli* (McQuestin et al. 2009) and *L. monocytogenes* and *E. coli* (Zhang et al. 2010).

## 2.2 Materials and Methods

### 2.2.1 Bacterial Strains and Media

This study initially determined inactivation kinetics at pH 3.5 and $a_w$ 0.90 of three *L. monocytogenes* strains (ATCC 19115, LO28 and 70-1700). Inactivation of those three strains and strains Scott A and FW035-0035 were also studied at pH 4.5 and $a_w$ 0.93 or 0.95. All strains were obtained from the School of Agricultural Science Culture Collection (University of Tasmania). Detailed descriptions of bacterial strains are provided in Table A.1 in Appendix A. *L. monocytogenes* strains were maintained in Nutrient Broth (Oxoid CM1, Adelaide, SA, Australia) with 30% glycerol (Sigma-Aldrich, Melbourne, VIC, Australia), and stored at -80°C. Frozen cultures were recovered on Brain Heart Infusion agar (BHA; Oxoid CM225 to which 15g agar was added per litre) at 37°C for 24h.

### 2.2.2 Inactivation of *L. monocytogenes* in response to low water activity (0.90) in combination with low pH (3.5) at 5, 10, 15, 25, 35 and 45ºC

#### *2.2.2.1 Preparation of Stationary Phase Populations of* L. monocytogenes

To prepare experimental inocula, *L. monocytogenes* cells were removed from the surface of thawed stock culture (stored at -80°C) using a sterilised inoculation loop, plated to BHA supplemented with 0.1% sodium pyruvate (Sigma, USA) (BHAP) and incubated for 24 (±0.25) hours at 37 (±0.5) °C. Five colonies of each *L. monocytogenes* strain on BHAP were inoculated to 100 ml Tryptone Soya Broth containing 0.6% yeast extract (TSB-Ye) in a 250 ml Erlenmeyer flask and incubated statically at 37 (±0.5) °C for 24 (±0.25) hours to achieve a population with viable count of approximately 9.0 log CFU per ml.

#### *2.2.2.2 Preparation of Low Water Activity and Low pH Broth*

A measured amount (14.22 g) of NaCl (Ajax Finechem, Taren Point, NSW, Australia) was added to 100 ml of TSB in a 250 ml Erlenmeyer flask to provide broth with an $a_w$ of 0.900 (±0.003). The amount of NaCl required to achieve the desired $a_w$ was estimated from the tables of Chirife and Resnik (1984) taking into account the intrinsic $a_w$ of the media used. The pH of the broth was aseptically adjusted to 3.50 (±0.05) with 10 M hydrochloric acid (Merck, Melbourne, VIC,

Australia) and measured using a pH meter (Model 250A, Orion Research, Boston, MA, USA). The mineral acid HCl was chosen as the acidulant, rather than lactic acid, to facilitate rapid amendment of the basal media without affecting broth characteristics such as $a_w$ and the amount of precipitate. Flasks were incubated at 5, 10, 15, 25, 35 or 45°C in a shaking water bath (Ratek Instruments, Boronia, VIC, Australia) for several hours to equilibrate to experimental temperature prior to inoculation.

#### *2.2.2.3 Harvesting* L. monocytogenes *and Inoculation into Broth*

An aliquot (5 ml) of stationary phase population (2.2.2.1) of each *L. monocytogenes* strain was transferred to a 15 ml sterile tube. The strains were not mixed. Cells were pelleted by centrifugation at 5000 rpm for 10 minutes at room temperature in a Universal 16A centrifuge. The supernatant was decanted and 2 ml of low-$a_w$/pH broth, prepared as described in section (2.2.2.2), was used to resuspend the cell pellet. The cell suspension was then added to 78 ml of low-$a_w$/pH broth to make up 80 ml. Each broth contained a single strain of *L. moncytogenes*.

#### *2.2.2.4 Enumeration of Viable Cells and Construction of Survival Curves*

The viability of each population was estimated by culture-based enumeration immediately prior to, and at regular intervals throughout, the low-$a_w$/pH treatment. Specifically, 100 µl aliquots were removed and serially diluted in 0.1% bacteriological peptone supplemented with 0.85% NaCl (peptone water). Samples (50, or 250 µl) were surface plated using a spiral plater (Autoplate 4000 Spiral Biotech Inc., Bethesda USA) onto BHAP. Plates were incubated for 24 (±0.25) hrs at 37 (±0.5) °C and colony forming units (CFU) were counted. Inactivation curves were constructed by plotting $\log_{10}$CFU.ml$^{-1}$ against time. When viable cells could not be detected in a 50 µL volume of an undiluted sample, an undiluted 250 µl sample was taken the next day. Where a 250 µl volume was plated and no colonies resulted, the number of viable cells was plotted as 0 log CFU per ml. Inactivation kinetics were assumed to be log-linear, therefore, the rate of inactivation for each strain was estimated by linear regression analysis using Microsoft® Excel. Where inactivation curves appeared to be multiphasic the rate of inactivation of each phase was calculated. Inactivation rates were then plotted on Arrhenius plots (*ln*(inactivation

rate) *vs.* 1/temperature[K]) to determine the temperature dependence of inactivation rate. The strength of the influence of temperature on the inactivation rate was estimated by calculating the correlation coefficient ($R^2$) and the root mean squared error (RMSE).

### 2.2.3 Inactivation of *L. monocytogenes* in response to low water activity (0.93 or 0.95) in combination with low pH (4.5) at 15ºC

Growth limits predictive models for *L. monocytogenes* developed by the University of Tasmania Food Microbiology Research Group and based upon Tienungoon et al (2000) were used to determine combinations of pH and $a_w$ at 15°C that were predicted to prevent growth of *L. moncytogenes*. The models used were originally developed for *L. monocytogenes* strains Scott A and L5. At pH 4.5, $a_w$ 0.95, the L5 model predicted 20% probability of growth while the Scott A model predicted 64% probability of growth. At $a_w$ 0.93 the predictions were 2% and 31% respectively. The model calculates the probability of growth of *L. monocytogenes* for different combinations of temperature, pH and $a_w$. Preparation of cultures and broths was as per sections 2.2.2.1, 2.2.2.2 and 2.2.2.3. NaCl (Ajax Finechem, Taren point, NSW, Australia) and 10 M hydrochloric acid (Merck, Melbourne, VIC, Australia) were used to prepare TSB with pH of 4.5 and $a_w$ of either 0.93 or 0.95. These flasks were incubated at 15°C only in a shaking water bath (Ratek Instruments, Boronia, VIC, Australia). Population viability was determined by enumeration of cells, and survival curves constructed, as described in Section 2.2.2.4. Additionally, the mean inactivation rate and standard deviation for each strain was calculated for each combination of pH and $a_w$ and differences were assessed by Student's t-test, using Microsoft® Excel. Differences were considered to be significant when $p < 0.05$.

### 2.2.4 Comparison of Arrhenius models

The Arrhenius model generated for non-thermal inactivation of *L. monocytogenes* in this study was compared with analogous non-thermal Arrhenius models generated by Zhang et al (2010) for *L. monocytogenes* and *E. coli* and McQuestin et al (2009) for *E. coli*.

To assess whether the rates of inactivation responses were significantly different between species or models used in this study and those of Zhang et al (2010), Arrhenius models were fitted to each dataset separately or to the combined dataset or to the combined data set using a common estimate of the slope but with different intercepts for each data set. The significance of the difference in goodness-of-fit of each model was evaluated by calculating the F ratio.

Specifically, temperature *vs.* inactivation rate datasets were transformed into 1/(absolute temperature) and *ln*(inactivation rate). These data were fitted to a straight line and the slope and intercept determined using the "SLOPE' and 'INTERCEPT' functions in Microsoft ® Excel software. The Solver add-in for optimization in Microsoft ® Excel was then used to determine the best values for the parameters of the model by iterative estimation, by minimizing the sum of the squares of the residuals (i.e., differences between the observed and modelled values of *ln*(inactivation rate). This latter estimation was done for consistency because Solver, needed for subsequent model fitting, does not produce the exact solution for the parameter values. Results from Solver were compared to the explicit solution (i.e., using the "SLOPE' and 'INTERCEPT' functions) and were generally found to agree to at least three significant figures.

Solver was then used to derive the best-fit parameters of the model describing the combined data with two lines allowing each data-set a discrete intercept but with the lines forced to have the same slope and, finally, by describing the combined data with a single line, i.e., one slope and one intercept value. Thus three models were fitted to enable the significance of differences between the data-sets, representing different organisms or different inactivation environments, to be determined. These are:

i.) **Model 1**: the data from each trial were fitted with a separate line, requiring the estimation of four parameters values, namely the slope and intercept for the line-of-best fit through each data-set. This model assumes that the treatments, or organisms, produce different responses. T his model requires the estimation of four parameters, *viz*, two slopes and two intercepts

ii.) **Model 2**: a model in which the lines of best fit through each data-set are forced to have the same slope but the lines through each data set can have different intercepts (i.e., parallel lines that may differ in position on the graph). This model assumes that temperature has the same relative effect on the organisms, or in different environments, but that the absolute rate of inactivation may be different. Model 2 requires the estimation of three parameters, *viz*, one slope and two intercepts.

iii.) **Model 3**: is the single straight line model that best describes the combined data from both organisms, or treatments. This model assumes that the inactivation rate at a given temperature is the same, irrespective of organism or of other environmental conditions. Model 3 requires the estimation of two parameters, *viz*, one slope and one intercept.

By comparing the goodness-of-fit (i.e. the magnitude of the sum of the squares of the residuals) of each model with the number of parameters that must be estimated for the model, the significance of the difference between the models can be tested. Thus, if Model 1 (which has more parameters) does not produce a significantly better description of the data than Model 2, it can be concluded that the relative effect of temperature on the rate of inactivation is the same for the organisms, or in the environments, being compared. Similarly, if Model 1 does not produce a significantly improved goodness- of-fit compared to Model 3 (which has half as many parameters with which to describe the data), it can be concluded that bacterial species, or the environments being compared, do not have, or cause significantly different inactivation rates in inimical environments in which temperature *per se* is not lethal.

The significance of difference in the goodness-of-fit is determined using an F statistic, calculated from the sum of squares of residuals and degrees of freedom involved in the two models being compared, where the degrees of freedom is the difference between the number of parameters in the model and the number of data being modelled.

The F-ratio is given by the following equation:

$$\text{F-ratio} = \frac{(SS_1 - SS_2)/(DF_1 - DF_2)}{(SS_2/DF_2)} \qquad \text{(Equation 2.1)}$$

Where: $SS_1$ = sum of squares of residuals of the first model being compared, $SS_2$ = sum of squares of residuals of the second model being compared, $DF_1$ is the degrees of freedom of the first model (number of data in combined data set – number of parameters to be estimated) and $DF_2$ is the degrees of freedom of the second model being compared.

Essentially this test determines whether the increased number of model parameters results in a significantly improved goodness-of-fit. The significance of the F-ratio is determined by calculating the corresponding *p*-value, given the number of degrees of freedom. This was calculated using the "FDIST" function of Microsoft® Excel. $p \leq 0.05$ was used as the threshold for significance.

The same approach as described above was used to assess the validity of the assumption that the response is best described by an Arrhenius model, i.e., a straight line when plotted as 1/(absolute temperature) vs. *ln*(inactivation rate). In this case the first model for comparison was the 1/(absolute temperature) vs. *ln*(inactivation rate) data fitted to a straight line (i.e. two parameters to be estimated) and the second model was a quadratic equation (i.e. three parameters to be estimated) which can describe a curved line response.

### 2.2.5 Differential effects of temperature, pH and water activity on *L. monocytogenes* inactivation assessed by meta-analysis

#### *2.2.5.1 Search strategy and selection criteria*

Studies of the survival of *L. monocytogenes* in fermented dairy products, fermented meats or aqueous systems (broths) with levels of pH and/or $a_w$ that prevented growth of the pathogen were sought by computer-based searches of literature databases, namely ISI's Web of Knowledge, and ComBase (www.combase.cc). Searching used key words '*Listeria monocytogenes*' with words related to bacterial inactivation ('inactivation', 'survival', 'death', and 'viability') and 'cheese'. Additional studies were identified from reviews of reference lists of the original studies. ComBase records were searched using key words '*Listeria monocytogenes*' with parameters of

$a_w$ (where provided) ≥0.75 and pH of any value. Records were individually reviewed and accepted for use if the data indicated that inactivation of *L. monocytogenes* occurred. Data presented in this chapter as well as elsewhere in this thesis (Chapters 3 and 4), PhD theses, industry reports and unpublished data from members of the University of Tasmania's Food Microbiology Research Group were also included. Only studies that were written in English and contained data that allowed an estimate of inactivation rate at some temperature were included in the analysis. Some ComBase records had only one or two recorded observations and these were omitted since it was not possible to obtain an accurate inactivation rate from less than 3 data points.

#### *2.2.5.2 Data abstraction and determination of inactivation rates*

Where possible, the following information was collated for each study: *L. monocytogenes* strain(s), food or broth type, process and duration of process, temperature, pH, $a_w$, presence and concentration of other additives and *L. monocytogenes* viability data. For each source that included data describing the enumeration of *L. monocytogenes* over a time period where temperature was constant, a rate of inactivation of *L. monocytogenes* was estimated in the same way as described in Section 2.2.2.4. Where inactivation curves were genuinely multiphasic, i.e. were not described by a straight line and there was no reported evidence of environmental change during the time course of the experiment, the rate of inactivation during the slowest phase (as determined by eye) was calculated as in Section 2.2.2.4. This strategy was used to present the "worst case" scenario of pathogen inactivation in a food product.

#### *2.2.5.3 Statistical analysis*

To identify possible predictor variables for the inactivation of *L. moncytogenes*, inactivation rates were collated and fitted to models based on the predictor variables of temperature, pH and $a_w$. Data were transformed and plotted on Arrhenius coordinates and analysed using simple linear regression as described in Section 2.2.2.4. The strength of the relationships was determined and compared by calculation of the $R^2$ and the RMSE.

Inactivation at several temperatures was able to be determined for many of the studies cited. Inactivation rates derived from data from studies for temperatures in the range 0-42°C were fitted to an Arrhenius model. To determine different temperature effects by lethal and sub-lethal temperatures, the inactivation rate data set for temperatures above 42°C was expanded by calculating inactivation rates from studies of the viability of *L. monocytogenes* at 45-68°C in various media. A total of 52 inactivation rate estimates for temperatures above 45°C were selected from records found in the original search (Section 2.2.2.5).

The effect of pH on the inactivation of *L. monocytogenes* was analysed by taking the residuals of the fitted line (*ln*(inactivation rate) observed - *ln*(inactivation rate) predicted by the Arrhenius model) and plotting them against pH. Simple linear regression was used to identify relationships between the normalised data and pH. Similarly the effect of $a_w$ on the inactivation of *L. monocytogenes* was analysed by plotting the residuals of the *L. monocytogenes* inactivation model for temperature (i.e. Arrhenius model) against the final $a_w$ of the experimental system where it was available. A simple linear regression was fitted to the data set and $R^2$ value determined.

## 2.3 Results

### 2.3.1 Inactivation of *L. monocytogenes* in response to low water activity (0.90) in combination with low pH (3.5) at 5, 10, 15, 25, 35 and 45ºC

Using an aqueous broth model system, the inactivation kinetics for three strains of *L. monocytogenes* exposed to pH 3.5 and $a_w$ 0.90 and temperatures in the range 5 to 45°C were determined. A total of 18 inactivation curves were generated for the three strains and the inactivation curves of *L. monocytogenes* strain ATCC 19115 at each temperature (5- 45°C) are shown in Figure 2.1. It is clear from Figure 2.1 that the inactivation of *L. monocytogenes* strain ATCC 19115 at 5°C was faster (187 days) than its inactivation at 10°C (286 days). Curves for strains LO28 and 70-1700 are shown in Appendix B, Figures B1 – 2. It is evident from these graphs that the inactivation curves for both strains at 5 and 10°C overlap and appear very similar with strain LO28 inactivation taking 262.5 and 286 days respectively and strain 70-1700 inactivation taking 287 and 286 days respectively.

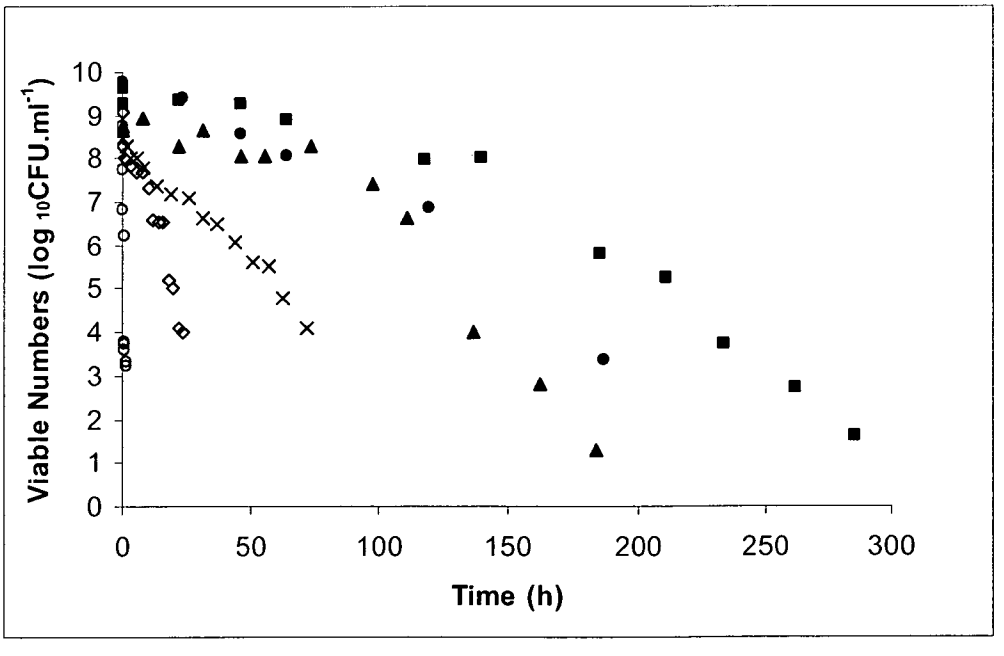


**Figure 2.1.** The inactivation curves for *L. monocytogenes* ATCC 19115 suspended in broth with pH 3.5 (HCL as acidulant) and $a_w$ 0.90 (NaCl as humectant) and incubated at 5°C (•), 10°C (■), 15°C (▲), 25°C (×), 35°C (◊ ), and 45°C (○).

Representative inactivation curves for each strain at 5, 25, 35, and 45°C are shown in Figure 2.2. Curves for the inactivation of all strains at 10 and 15°C are shown in Appendix B, Figures B3 - 4. Despite the variability between individual datasets, collectively the data at all temperatures suggests that there are no systematic differences, due to the strain, in the inactivation rate of *L. monocytogenes* at pH 3.50 and $a_w$ 0.90. In particular, with the exception of the 45°C data (figure 2.2d), the inactivation curves for each strain of *L. monocytogenes* vary over similar ranges for each temperature. At 45°C, however, it is apparent that at pH 3.50 and $a_w$ 0.900, the inactivation of *L. monocytogenes* appears to be more complex than at lower temperatures with the inactivation kinetics appearing to be biphasic in nature. The same response was observed by Zhang et al (2010) for the inactivation of *L. monocytogenes* under the same broth pH and $a_w$ conditions (data were derived in collaboration with the novel studies described in this Chapter). Those authors concluded that the inactivation of *L. monocytogenes* at 45°C represented uncontrolled experimental variation, possibly due to thermal inactivation occurring at 45°C, the upper temperature limit for growth of *L. moncytogenes*, and considered that the inactivation at 45°C was best characterised by the slower inactivation rate observed.

Table 2.1 shows the inactivation rates calculated by simple linear regression from the inactivation curves for each strain at all temperatures. The rates in Table 2.1 were plotted using Arrhenius coordinates as shown in Figure 2.3. The slopes, $y$-intercepts and $R^2$ for each individual strain are detailed in Table 2.2 and show that the data for each strain is well explained by the Arrhenius equation (average $R^2 = 0.85$). The $R^2$ for the whole data set (with all strains treated as one population) in Figure 2.2 is 0.84 indicating that temperature has a strong influence on the rate of non-thermal inactivation of *L. moncytogenes,* i.e., it explains 84% of the variance in the observed *ln*(inactivation rate) data. As such, temperature explains much more of the variance in the data than does *L. monocytogenes* strain, and the Arrhenius model provides a good description of the response. The RMSE is 0.718. At 45°C, however, all three *L. monocytogenes* strains exhibited biphasic or curvilinear inactivation kinetics as shown in Figure 2.2d. When these points were removed from the data set, the $R^2$ becomes 0.88 and the RMSE 0.298. Zhang et al (2010) observed an improvement in the fit of their Arrhenius model when inactivation data for *L. monocytogenes* at 45°C

data were removed also. Inactivation data at 5°C for each of the three strains appears to be anomalously fast as noted above. The rates of inactivation shown in Table 2.1 indicate that at 5 and 10°C rates of inactivation are almost identical. This was not observed by Zhang et al (2010) with *L. monocytogenes* under the same conditions and with one strain in common (ATCC 19115). When figure 2.3 is examined, the data points corresponding to inactivation experiments undertaken at 5°C appear to show a concave upward "tailing". With the removal of the 5 and 45°C data sets, the $R^2$ becomes 0.92 and the RMSE 0.243.

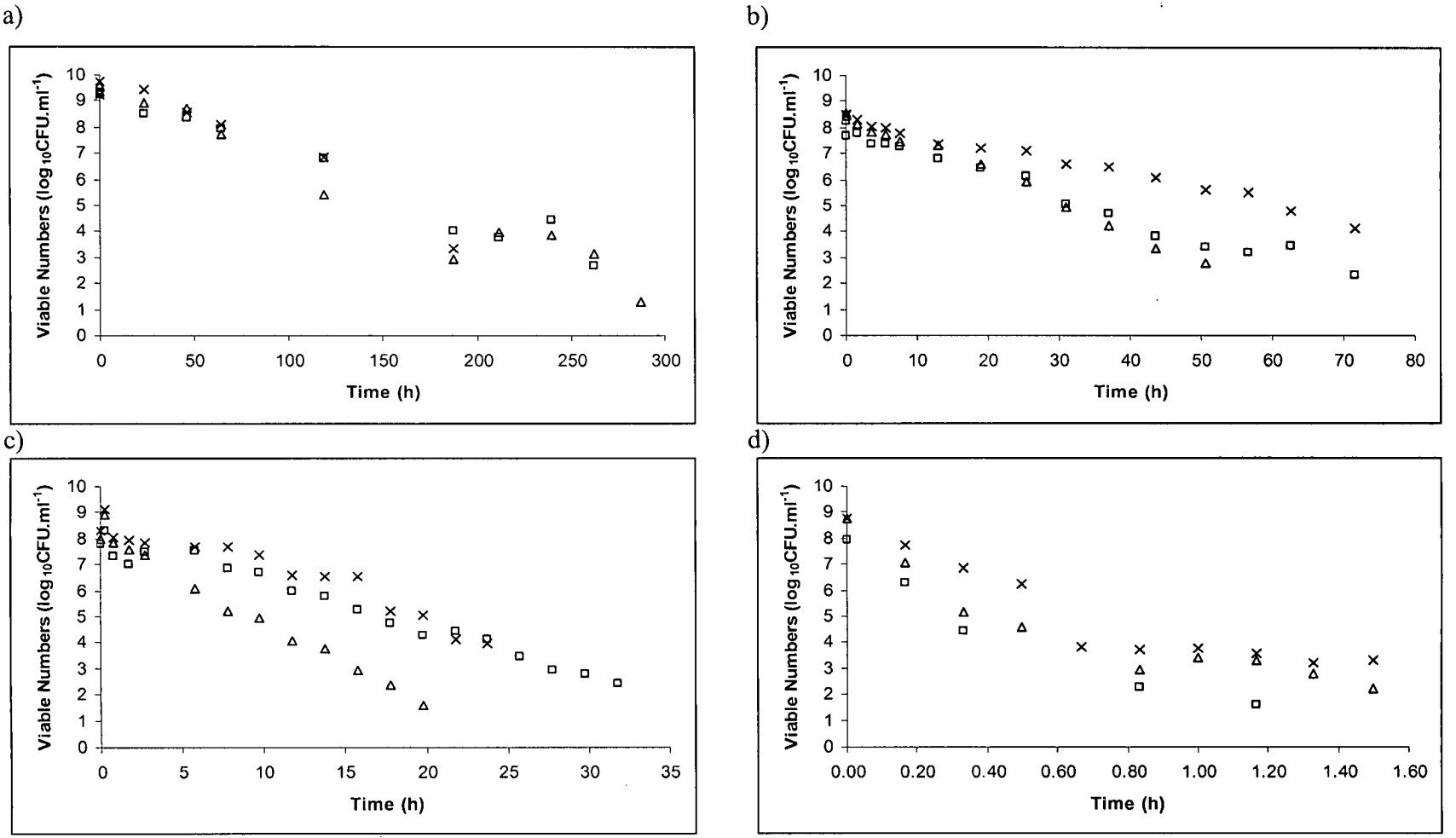


**Figure 2.2**. Representative non-thermal inactivation curves for *L. monocytogenes* ATCC 19115 (×), LO28 (□), and 70-1700 (Δ) suspended in broth with pH 3.5 (HCL as acidulant) and aw 0.90 (NaCl as humectant) and incubated at (a) 5°C, (b) 25°C, (c) 35°C, and (d) 45°C.

**Table 2.1.** Rate of inactivation ($log_{10}$CFU per hour) of *L. monocytogenes* in BHI at pH 3.5 and $a_w$ 0.90 (NaCl) at various temperatures.

| Temperature (°C) | *Inactivation Rate* ($log_{10}CFU.h^{-1}$) | | | |
|---|---|---|---|---|
| | *L. monocytogenes* strain | | | |
| | ATCC 19115 | 70-1700 | LO28 | *Mean (± SD)* |
| 5 | 0.0274 | 0.026 | 0.0274 | 0.027 (0.0008) |
| 10 | 0.0265 | 0.026 | 0.0234 | 0.025 (0.0017) |
| 15 | 0.0378 | 0.034 | 0.0309 | 0.034 (.035) |
| 25 | 0.055 | 0.1119 | 0.0798 | 0.082 (.0285) |
| 35 | 0.182 | 0.3539 | 0.1724 | 0.236 (.1021) |
| 45 | 2.3083 | 3.772 | 6.2959 | 4.125 (2.017) |

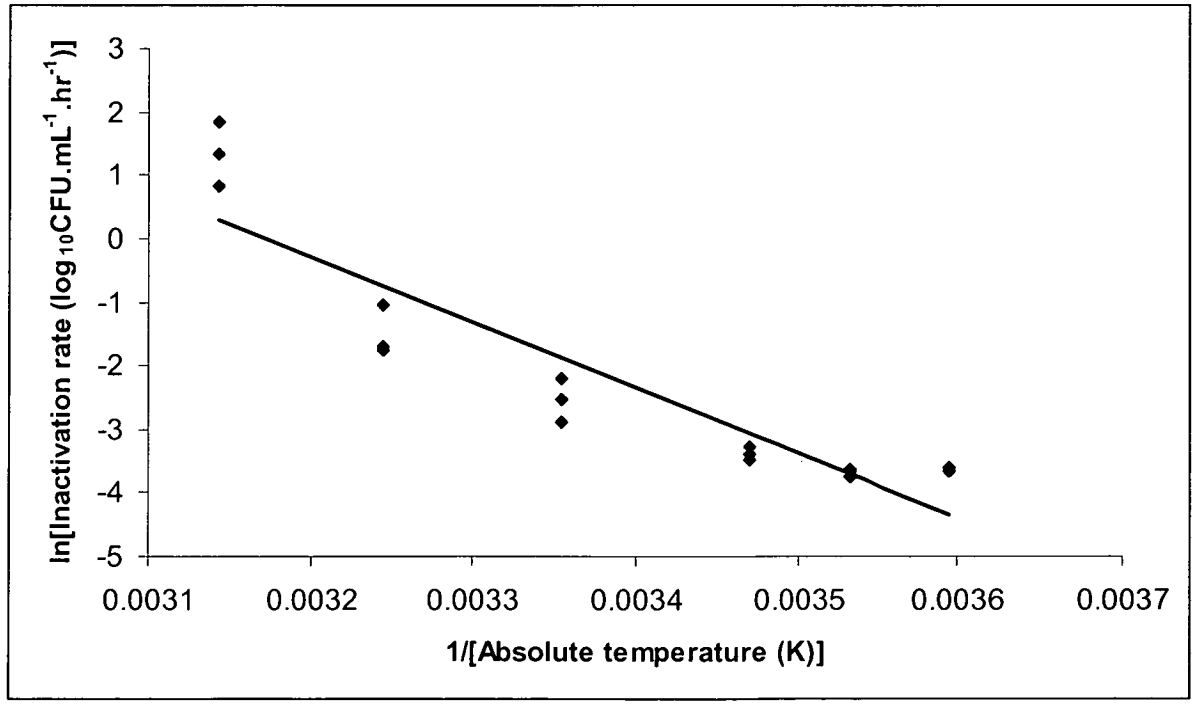


**Figure 2.3.** Arrhenius plot showing the effect of temperature (5-45°C) on the rate of inactivation of *L. monocytogenes* suspended in broth poised at pH 3.5 and $a_w$ 0.90. The regression equation fitted to the data is $y = -10248x + 32.512$ ($R^2 = 0.84$).

**Table 2.2.** Slope, *y*-intercept and correlation co-efficient of Arrhenius models for the rate of inactivation of *L. monocytogenes* strains in broth at pH 3.5 and $a_w$ 0.90

| Strain | Slope | *y*-intercept | $R^2$ |
|---|---|---|---|
| ATCC 19115 | -9037 | 28.255 | 0.83 |
| LO28 | -10927 | 34.828 | 0.80 |
| 70-1700 | -10782 | 34.525 | 0.91 |
| *Mean (±SD)* | *-10249 (±1051.8)* | *31.76 (±2.70)* | *0.85 (±0.06)* |
| *All data combined* | -10248 | 32.512 | 0.84 |

### 2.3.2 Inactivation of *L. monocytogenes* in response to low water activity (0.93 or 0.95) in combination with low pH (4.5) at 15ºC

A further 10 inactivation curves were generated for five *L. monocytogenes* strains (strains Scott A and FW035-0035 were not used in the experiments in 2.3.1) using an aqueous broth model acidified to pH 4.5 and with $a_w$ of either 0.93 or 0.95. The inactivation kinetics data are shown in Figure 2.4 and 2.5.

It is evident from Figures 2.4 and 2.5 that the two added strains, Scott A and FW035-0035, exhibit slower rates of inactivation at pH 4.5 and both $a_w$ 0.93 and 0.95 than either of the original three strains, ATCC 19115, LO28 and 70-1700. Figure 2.5 indicates that strain FW035-0035 is very slowly inactivated when the $a_w$ is 0.95, with population decline appearing to cease at approximately 350 hours. After almost 600 hours, the population numbers are still at ~6 logs.

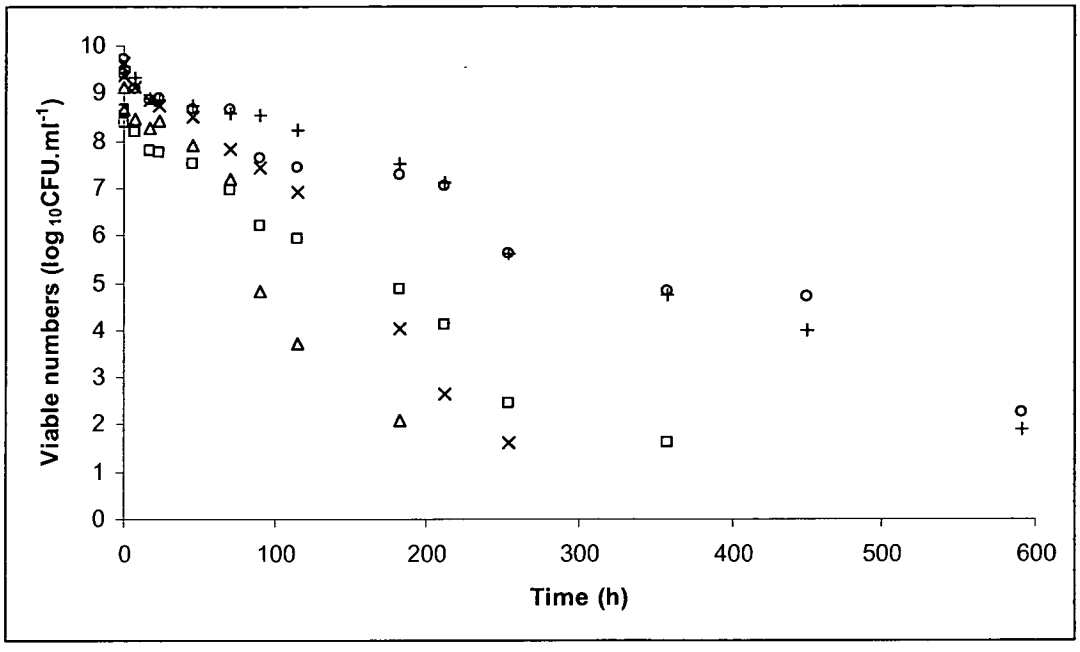


**Figure 2.4.** Non-thermal inactivation curves for *L. monocytogenes* ATCC 19115 (×), LO28 (□),70-1700 (Δ), Scott A (○) and FW035-0035 (+) suspended in broth with pH 4.5 (HCL as acidulant) and $a_w$ 0.93 (NaCl as humectant) and incubated at 15°C.

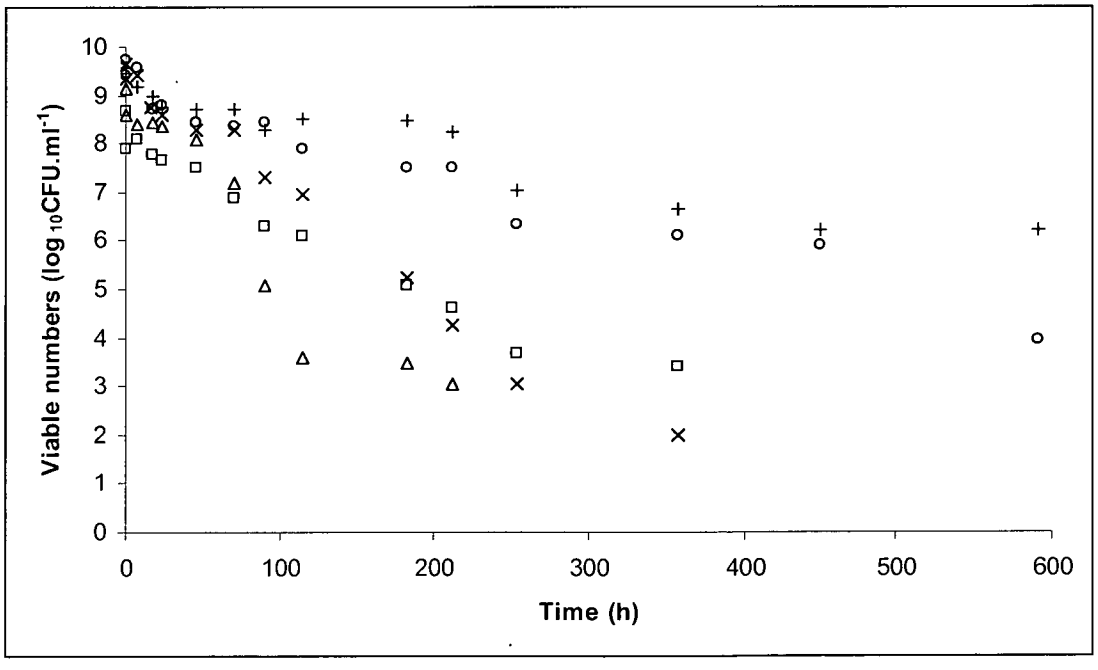


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**Figure 2.5.** Non-thermal inactivation curves for *L. monocytogenes* ATCC 19115 (×), LO28 (□),70-1700 (Δ), Scott A (○) and FW035-0035 (+) suspended in broth with pH 4.5 (HCL as acidulant) and $a_w$ 0.95 (NaCl as humectant) and incubated at 15°C.

Table 2.3 shows the inactivation rates calculated, by simple linear regression, from the inactivation curves for each strain at 15°C. The rates in Table 2.3 were plotted, together with the original inactivation rates from Figure 2.3, using Arrhenius coordinates as shown in Figure 2.6. The $R^2$ for the whole data set in the temperature range 5-45°C (with all strains treated as one population) in Figure 2.6 is 0.77. The inactivation rates for the three strains originally tested (ATCC19115, LO28 and 70-1700) of *L. monocytogenes* appear to systematically slower at 15°C when the pH and $a_w$ conditions are less extreme (Table 2.3). Inactivation rates for these three strains at pH 3.5 and $a_w$ 0.90, however, are not significantly different to the inactivation rates of the same strains at either $a_w$ 0.93 or $a_w$ 0.95 ($p = 0.16$ and $p = 0.57$ respectively, two-tailed t-test, unequal variances). There was no significant difference between the rates of inactivation for all five strains at $a_w$ 0.95 and 0.93 ($p = 0.38$, two-tailed t-test, unequal variances). However, the two strains Scott A and FW035-0035, introduced in the extra experiments at 15°C are more acid tolerant than the original three strains (Bowman 2009) and show slower inactivation in the conditions tested than the original three strains at the same conditions (Figures 2.4 and 2.5). Inactivation rates for the original three strains at $a_w$ 0.90 were significantly different to the inactivation rates of all five strains at $a_w$ 0.93 ($p < 0.05$). However, inactivation rates for the original three strains at $a_w$ 0.90 were not significantly different to the inactivation rates of five strains at $a_w$ 0.95 ($p = 0.11$). Analysis of the inactivation rates for the original three strains at $a_w$ 0.93 compared to those obtained for the two new strains at $a_w$ 0.93 showed a significant difference ($p = 0.03$) and likewise the difference was significant ($p < 0.05$) when comparing the three original strains at $a_w$ 0.95 with the two new ones at $a_w$ 0.95. Finally, when the inactivation rates for the original three strains at $a_w$ 0.93 and 0.95 are pooled and compared to the pooled rates at both $a_w$ values for the two new strains the differences are significant ($p = 0.005$) indicating clear strain differences. When the data for strains Scott A and FW035-0035 were removed from the Arrhenius model shown in Figure 2.6 the slope becomes -10698 and the $R^2$ is 0.83. When the inactivation rates for the 45°C data are also removed from the data set (as was done for the data in Figure 2.3), the $R^2$ becomes 0.78 and the slope is -6642.8. With removal of the anomalous 5°C data also the $R^2$ becomes 0.87 and the slope is -8310.

Examination of the Arrhenius plots in both Figure 2.3 and 2.6 suggests a concave upward curvature. A quadratic polynomial was fitted to the data shown in Figure 2.6 (for the original three strains only and *sans* points at 45°C) and the goodness-of-fit of the equation was compared to that for the linear model fitted to the same data. Calculating the F ratio (see S. 2.2.3.1) the quadratic model significantly ($p < 0.0005$) better describes the effect of temperature on the inactivation rate than a straight line. When the 5°C data were removed, however, the quadratic line did not provide significantly better ($p > 0.05$) description of the data than the straight line model.

**Table 2.3.** Rates of inactivation (log CFU.h$^{-1}$) at 15°C for *L. monocytogenes* at pH 4.5 and $a_w$ 0.93 or 0.95 or pH 3.5 and $a_w$ 0.90. Italicised strain names are the original strains used in the inactivation at pH 3.5 and $a_w$ 0.90.

| $A_w$ | *L. monocytogenes* strain | | | | | Mean | SD |
|---|---|---|---|---|---|---|---|
| | Scott A | FW035-0035 | *ATCC 19115* | *LO28* | *70-1700* | | |
| 0.93 (pH 4.5) | 0.0115 | 0.0126 | 0.0309 | 0.0200 | 0.0415 | 0.0233 | 0.0128 |
| 0.95 (pH 4.5) | 0.0086 | 0.0059 | 0.0215 | 0.0166 | 0.0326 | 0.0170 | 0.0096 |
| 0.90 (pH 3.5) | - | - | 0.0378 | 0.03090 | 0.0340 | 0.0343 | 0.00282 |

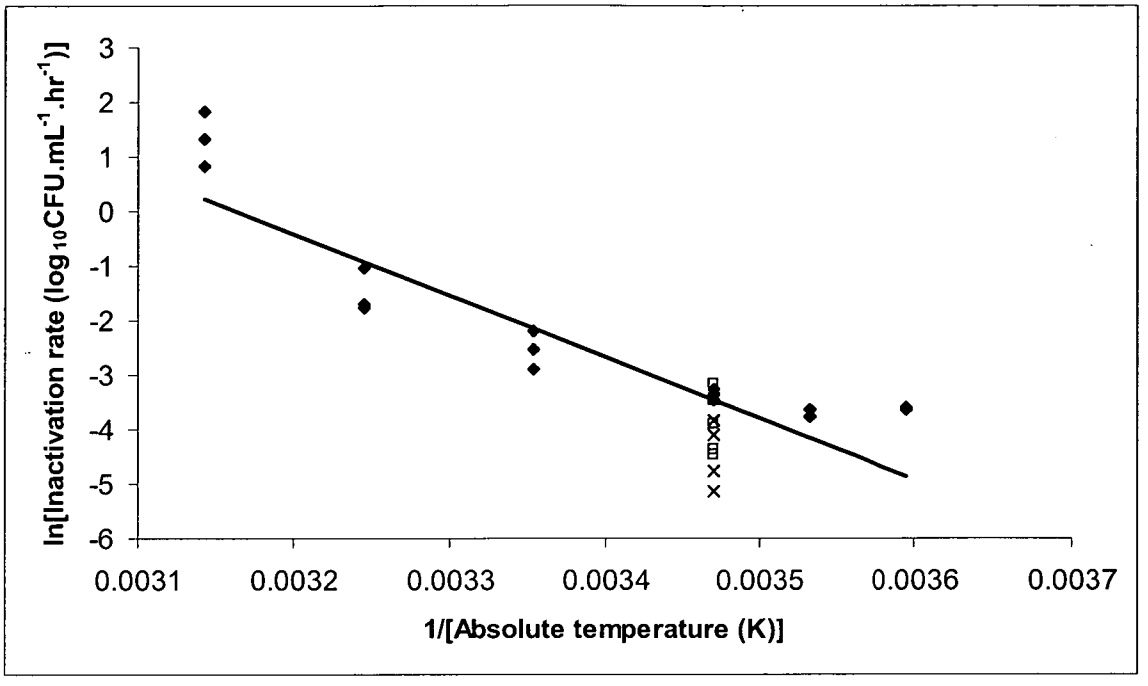


**Figure 2.6.** Arrhenius plot showing the effect of temperature (5-45°C) on the rate of inactivation of *L. monocytogenes* suspended in broth with pH 3.5 and $a_w$ 0.90 (♦) or pH 4.5 and $a_w$ 0.93 (□) or pH 4.5 and $a_w$ 0.95 (×). The regression equation fitted to the data is $y = -11220x + 35.47$ ($R^2 = 0.77$).

### 2.3.3 Model comparison

Figure 2.7 compares the Arrhenius model generated in Section 2.3.2 (without the two new strains due to their systematically slower inactivation rates) with the models of Zhang et al (2010), for *L. monocytogenes* and *E. coli* inactivation data in broth at pH 3.5 and $a_w$ 0.90, the models of McQuestin et al (2009), derived from studies of *E. coli* inactivation in fermented meats and analogous aqueous systems, and McQuestin (2006), using *E. coli* inactivation rate data derived from systematic and well-controlled studies involving the same strains of *E. coli* in salami and analogous (matched for pH, temperature, water activity, lactic acid etc) broth systems. Data from Zhang et al (2010) were derived in collaboration with the novel studies described in this Chapter.

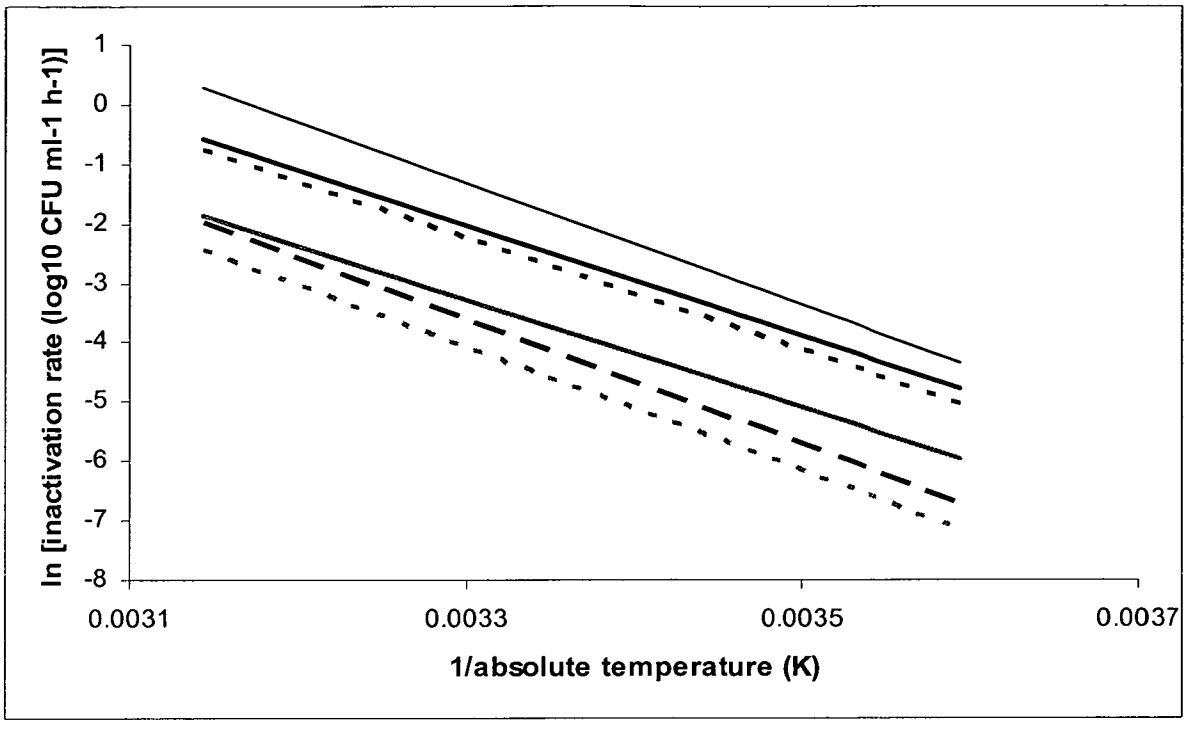


**Figure 2.7.** Comparison of the Arrhenius models for the non-thermal inactivation of vegetative bacteria at 5 to 45°C. The various models describe the inactivation of i) *L. monocytogenes* (solid fine, upper black line) in broth at pH 3.5 and $a_w$ 0.90 obtained during the current study; ii) and iii) *L. monocytogenes* (solid, black line) and *E. coli* (dashed, black line) in broth at pH 3.5 and $a_w$ 0.90 (Zhang et al. 2010); iv) *E. coli* inactivation in a salami product (dashed, grey line) and v) an analogous broth system (solid, grey line) (McQuestin et al. 2006); and vi) *E. coli* inactivation observed in a wide variety of fermented meat products and analogous aqueous systems using various strains and with different combinations of hurdles (large dashed, black line) (McQuestin et al. 2009).

The slope of the Arrhenius plots, which describes the relative effect of temperature on inactivation rate, appears to be consistent between the different studies and the two bacterial species initially supporting the hypothesis that the relative effect of temperature on the non-thermal inactivation of vegetative bacteria is not species-dependent. It is apparent, however, that there are systematic differences between the absolute rates of inactivation from the different studies and between inactivation rates derived from broth studies and those derived from fermented meat studies, with inactivation rates in aqueous broth systems being consistently faster than those derived in food based systems (in this case fermented meats).

Despite the differences in species, strain, media and conditions used to generate the models presented in Figure 2.7 the $R^2$ in all cases are ≥60% indicating that temperature accounts for over 60% of the variance in the observed *ln* inactivation rate. In the model comparison, it is evident that the slopes of all the datasets are similar. The raw inactivation rate data from the studies by McQuestin (2006) and McQuestin et al (2009) were not available to undertake statistical analysis of those models in comparison to other models shown in Figure 2.7.

A comparison between the inactivation data for *L. monocytogenes* derived from the current study and the inactivation data for both *L. monocytogenes* and *E. coli* from the work of Zhang et al (2010) was undertaken (see Section 2.3.3.1). The data from this study included the extra inactivation rate data at 15°C (pH 4.5 and $a_w$ 0.93 and 0.94) for the original three strains considered only. Data for 45°C were omitted for the reasons described earlier, and in Zhang et al (2010), and are considered in greater detail in the Section 2.4 . Zhang et al (2010) found no significant differences between the inactivation rates of *L. monocytogenes* and *E. coli* except at 25°C and that the apparent difference at 25°C was mostly due to an unusually consistent set of replicates. Statistical analysis of the rates of inactivation between *L. monocytogenes* from the current study and those from Zhang et al (2010) showed that there was a significant difference ($p < 0.05$) between the slopes of the fitted equations of the Arrhenius models. The relationship between the fitted models for both data sets is shown in Appendix B Figure B.5. When the same comparison was made, but excluding inactivation rates at 5°C, the difference between the slopes of the fitted lines was not significant (*see* Appendix B Figure B.6).

### 2.3.4 Investigation of differential effects of temperature, pH and water activity on *L. monocytogenes* inactivation by meta-analysis

#### *2.3.4.1 Data set*

A total of 45 relevant studies relating to the inactivation of *L. monocytogenes* in foods and analogous aqueous systems were identified. Individual reports included varying numbers of datasets, but yielded 1195 individual inactivation rates for temperatures in the range of 0 to 68°C as summarised in Table 2.6. Most of the studies allowed the calculation of multiple inactivation rate estimates and collectively they encompassed more than 100 *L. monocytogenes* strains, pH ranging from 2.7 to 7.4 and $a_w$ in the range 0.78 to 0.99. These data were compiled and analysed to quantify the patterns of *L. monocytogenes* inactivation in environments relevant to cheese.

#### *2.3.4.2 Effect of temperature*

The effect of temperature on *L. monocytogenes* inactivation was determined by plotting on Arrhenius coordinates and analysis by simple linear regression (Figure 2.8).

The $R^2$ for the Arrhenius equation fitted to the *L. monocytogenes* data in the temperature range of 0-68°C is 0.54 indicating that temperature explains 54% of the variance in the observed *ln*(inactivation rate) data.

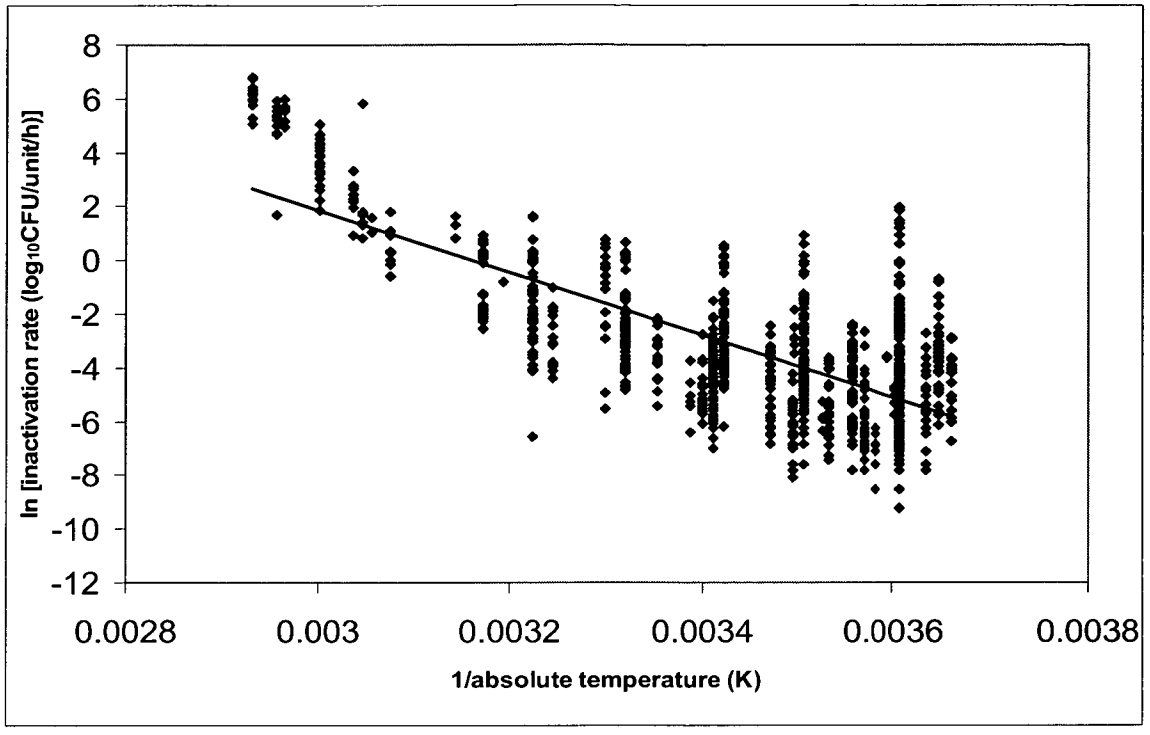


**Figure 2.8.** Arrhenius plot showing the effect of temperature (0-68°C) on the rate of inactivation of *L. monocytogenes* in foods and analogous aqueous systems (♦). The regression equation fitted to the data is y = -11660x + 36.872 (R2 = 0.54). The data encompass pH ranging from 2.7 to 7.4 and aw ranging from 0.78 to 0.995.

**Table 2.6**: Summary of studies and datasets included in meta-analysis of *L. monocytogenes* inactivation in cheese. The number of *L. monocytogenes* strains and the matrix used are summarised along with the number of inactivation rates derived for the specified ranges of temperature, pH and $a_w$.

| ComBase Reference Details | Source | # strains | Food or broth type | # rates | Temp. ( C)[a] | pH[a] | aw[a] |
|---|---|---|---|---|---|---|---|
| *FSA-CCFRA T165 | ComBase 2008 | 3 | Tryptic Phosphate Broth (TPB) | 9 | 2-15 (5) | 4-4.5 (2) | 0.997 (1) |
| *FSA-CCFRA T166 | ComBase 2008 | 3 | TPB | 7 | 2-10 (4) | 4-4.5 (2) | 0.997 (1) |
| *FSA-CCFRA T259 | ComBase 2008 | 3 | TPB | 100 | 0-20 (7) | 3.5 -7 (5) | 0.793-0.971 (5) |
| ElShenawy_90a | ComBase 2008 | 1 | Tryptose Broth (TB) and pasteurised milk | 6 | 13-35 (2) | 3.61-4.32 (4) | - |
| FSA-CCFRA T285 | ComBase 2008 | 1 | TPB | 24 | 60-68 (3) | 4.2-4.5 (2) | 0.943-0.997 (4) |
| FSA-IFR T113 | ComBase 2008 | 1 | Tryptone Soya Broth (TSB) | 2 | 20 (1) | 4.5-4.7 (2) | 0.997 (1) |
| Yousef_90a | ComBase 2008 | 2 | Parmesan cheese | 6 | 12.8 (1) | - | - |
| FSA-IFR T288 | ComBase 2008 | 1 | TSB + 1% glucose + 0.3% yeast extract | 3 | 20 (1) | 4.3-4.5 (2) | 0.997 (1) |
| *FSA-IFR T289 | ComBase 2008 | 1 | TSB + 1% glucose + 0.3% yeast extract | 90 | 1-20 (5) | 3.5-5 (8) | 0.997 (1) |
| *FSA-IFR T292 | ComBase 2008 | 1 | TSB+ 1% glucose + 0. 3% yeast extract + NaCl | 85 | 1-20 (6) | 3.4-4.5 (7) | 0.935-0.986 (4) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *FSA-IFR T412 | ComBase 2008 | 1 | TSB + 1% glucose + 0.3% yeast extract | 51 | 1-20 (5) | 3.5-4.5 (7) | 0.935-0.997 (3) |
| Holliday_03 | ComBase 2008 | 6 | sweet whipped unsalted butter, light salted butter, or yellow fat spread | 12 | 4.4 - 21 (2) | 4.05 - 4.58 (3) | - |
| Hwang_05 | ComBase 2008 | 5 | mayonnaise | 4 | 4 -8 (2) | 4.5 (1) | - |
| *Juneja_99c | ComBase 2008 | 4 | simulated beef gravy | 16 | 55 - 65 (3) | 4 (1) | 0.964-0.997 (3) |
| Larson_96 | ComBase 2008 | 5 | coleslaw or cottage cheese | 12 | 4-12 (2) | 3.8-4.5 (2) | - |
| Lou_97 | ComBase 2008 | 1 | TSB with 0.6% yeast extract (TSB-Ye) | 4 | 30 (1) | 3.5 (1) | - |
| *Buchanan_90 | ComBase 2008 | 1 | TPB | 1 | 37 (1) | 4.5 (1) | 0.997 (1) |
| *Buchanan_94a | ComBase 2008 | 3 | Brain Heart Infusion Broth (BHIB) | 260 | 4-42 (6) | 2.7-4.8 (17) | 0.856-0.997 (6) |
| *Buchanan_95 | ComBase 2008 | 3 | BHIB | 135 | 4-42 (6) | 3.3-4.8 (14) | 0.856-0.997 (8) |
| *Buchanan_97a | ComBase 2008 | 3 | BHIB | 29 | 4-42 (5) | 3-5 (7) | 0.856-0.997 (6) |
| | (Morgan et al. 2001) | 1 | Blue goat cheese | 2 | 13 (1) | - | - |
| | (Yousef and Marth 1988) | 2 | Colby cheese | 6 | 4 (1) | 5-5.19 (5) | - |
| | (Larson et al. 1999) | 6 | Cheese brine | 45 | 4 (1) | 4.9-5.3 (3) | - |
| | (Boussouel et al. 2000) | 1 | Skim milk | 10 | 25 (1) | 6.4 (1) | 0.99 (1) |
| | (Papageorgiou and Marth 1989b) | 2 | Feta cheese | 6 | 4 (1) | 4.3 (1) | 0.974-0.977 (4) |
| | (Papageorgiou and | 2 | Blue cheese | 6 | 10.5 (1) | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Marth 1989a) | | | | | | |
| * | (Gahan et al. 1996) | 1 | TSB-Ye, natural yogurt, cottage cheese, whole fat Cheddar, low fat Cheddar, orange juice, salad dressing | 20 | 4-37 (3) | 3-5.25 (6) | - |
| | (Koutsoumanis et al. 2003) | 5 | Trypticase Soy Broth (TSB) without glucose | 7 | 30 (1) | 3.5 (1) | - |
| | (Jørgensen et al. 1995) | 1 | TPB | 5 | 60 (1) | - | - |
| | (Whiting and Masana 1994) | 3 | Simulated UCFM | 3 | 37 (1) | 4.3-5 (3) | - |
| | (Bachmann and Spahr 1995) | 1 | Semihard cheese | 1 | 12 (1) | - | - |
| | (Panari et al. 2004) | 1 | Parmigiano-Reggiano cheese | 1 | 40 (1) | - | - |
| | (Degenhardt and Sant'Anna 2007) | 1 | Low acid Italian sausage | 3 | 13 (1) | - | - |
| | Bignell et al unpublished | ? | Lactic and fresh curd cheese | 4 | 4-20 (2) | 4.1-5.6 (2) | - |
| | (Ryser and Marth 1987a) | 3 | Cheddar cheese | 18 | 6-13 (2) | 5.04-5.09 (6) | - |
| * | (Shahamat et al. 1980) | 1 | TSB | 14 | 4-37 (3) | 7.4 (1) | - |
| | (Kroll and Patchett 1992) | 1 | Yeast Dextrose Broth (YDB) or Nutrient Broth (NB) | 2 | 37 (1) | 3 (1) | - |
| * | (O'Driscoll et al. | 3 | TSB-Ye, sodium phosphate | 16 | 22-54 (3) | 3.5-7 (2) | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1996) | | buffer with NaCl, phosphate buffered saline (PBS), ethanol, crystal violet, hydrogen peroxide | | | | |
| | (El-Shenawy and Marth 1990) | 1 | TB and milk | 12 | 13-35 (2) | 3.61-4.28 (9) | - |
| | (Faleiro et al. 2003) | 1 | Trivett and Meyer medium ( TM) | 2 | 30 (1) | 7.3 (1) | - |
| | (Benkerroum et al. 2003) | 1 | Raw sausage (merguez) | 4 | 30 (1) | - | - |
| * | (Ahamad and Marth 1989) | 2 | TB | 51 | 7-13 (4) | 3.6-4.5 (6) | - |
| * | (Casadei and al 1998) | 2 | TSB, half cream, double cream and butter | 40 | 52-68 (5) | - | 0.952-0.997 (3) |
| * | This thesis (Chpt 2) | 5 | TSB-Ye | 28 | 5-45 (6) | 3.5-4.5 (2) | 0.90-0.95 (3) |
| * | This thesis (Chpt 3) | 3 | Semihard cows milk cheese (raw and pasteurised) | 12 | 4-25 (5) | - | - |
| * | This thesis (Chpt 4) | 3 | Blue ewe's milk cheese (raw) | 21 | 4-25 (6) | - | - |
| **Total #** | | | | | **Overall range** | | |
| | | >102[b] | | 1195 | 0-68 | 2.7-7.4 | 0.793-0.99 |

[a] Number of distinct temperature, pH or $a_w$, values for which inactivation rate was determined is given in parentheses.

[b] Exact number unknown because strain was unspecified in some studies

- Not described

* Studies individually fitted to Arrhenius coordinates (Section 2.3.4.2).

Figure 2.9 compares rates of inactivation at 0-42°C and 45-68°C. At temperatures greater than 45°C, the slope of the linear regression fitted to the data on an Arrhenius plot is increased compared to the slope of the regression at temperatures within the physiological range for *L. monocytogenes* (0-42°C). Figure 2.9 also illustrates the changing effect of temperature on the inactivation of *L. monocytogenes* at temperatures greater than 42°C.

The Arrhenius model fitted to the 0-42°C data is given by the equation:

$$ln[\text{inactivation rate } (\log_{10}\text{CFU/unit/h})] = 22.433 - 7531.2 \times [1/\text{temperature (K)}]$$
$$(\pm 1.64 \text{ RMSE}) \quad \text{(Equation 2.2)}$$

The $R^2$ for this equation is 0.24, i.e., temperature explains only 24% of the variance in the observed *ln*(inactivation rate) data for this temperature range.

To further test the influence of non-lethal temperature on *L. moncytogenes,* the entire dataset was disaggregated into the individual studies from which the inactivation rates were derived. The studies that provided inactivation rates at three or more temperatures were separately fitted to an Arrhenius model (data not shown). Those studies are indicated by an asterisk in Table 2.6. Some of the datasets from ComBase originated from one research body (FSA-IFR and FSA-CCFRA) and were not reported in the peer-reviewed literature. When the FSA data (treated as one dataset) was plotted on Arrhenius coordinates the slope was -688.29 and the $R^2$ = 0.0016. It was decided, therefore, to analyse the datasets that originated from the University of Tasmania's Food Microbiology Research Group (of which this dataset is one) and from the refereed literature only. When the non-refereed datasets were removed from the 0-42°C plot in Figure 2.9, the Arrhenius model had a slope of 8323 and an $R^2$ = 0.2648. Disaggregation of studies from the refereed literature also revealed that datasets from (Buchanan and Phillips 1989; Buchanan et al. 1994; Buchanan and Golden 1995; Buchanan et al. 1997) were the only datasets that did not show inactivation that was dominated by temperature. The data from the Buchanan group were very different from all the other datasets used. The Arrhenius model for the Buchanan datasets has a positive slope of 0.0387 and an $R^2$ of 0.08.

When these data were removed from the refereed literature only, the resulting Arrhenius model had $R^2$ of 0.4499.

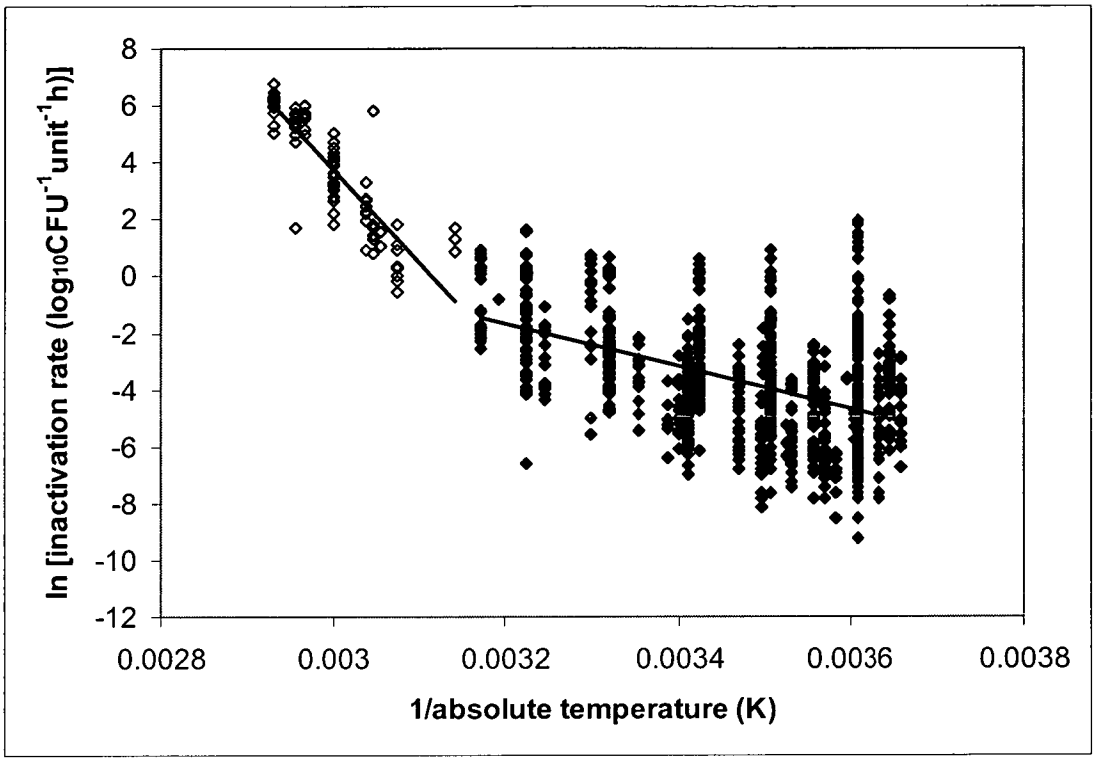


**Figure 2.9.** Arrhenius plot showing the changing effect of temperature on the rate of inactivation of *L. monocytogenes* for temperatures above (◊) and below (♦) 45°C which is the maximum temperature for growth of *L. monocytogenes*. The regression equation fitted to the data for temperatures above 42°C is $y$ = -32437x + 106.06 ($R^2$ = 0.76) and below and including 42°C is $y$= -7531.2x + 22.433 ($R^2$ = 0.24).

The studies used in the 0-42°C plot in Figure 2.9 were separated into studies undertaken in aqueous broth systems and those in foods and plotted on Arrhenius coordinates (data not shown). Data from broth studies had a slope of -6040, $y$ intercept of 17.546 and an $R^2$ = 0.1848. Data from studies undertaken in foods (see Table 2.6 for the range of foods) had a steeper slope of -10849, $y$ intercept of 32.804 and $R^2$ = 0.4175

As was also noted in the inactivation data for *L. monocytogenes* in broth (Figures 2.3 and 2.6), the *ln*(inactivation rate) response to temperature from 0-42°C in Figure 2.9 appears to be somewhat curvilinear and a better-fitting model may be available to better explain the data. Systematic removal of data from lower temperatures equated

with a systematic change in the slope indicating a continuous curve over the whole temperature range rather than an effect specifically occurring at low temperatures (data not shown).

### *2.3.4.3 Effect of pH*

The *ln*(rate of inactivation) data of *L. monocytogenes* for temperatures in the range of 0 - 42°C were normalised for the effect of temperature using the Arrhenius model given by Equation 2.1 i.e. by subtracting the *ln*(inactivation rate) predicted by the simple Arrhenius equation from that actually observed. The resultant residuals were plotted against pH (where available) as shown in Figure 2.10. The pH explained 21% ($R^2$ = 0.2142) of the over- or under-prediction of the Arrhenius model. When the non-refereed datasets were removed from the 0-42°C plot shown in Figure 2.10, the pH explained 25% ($R^2$ = 0.2509) of the over- or under- prediction of the Arrhenius model. Similarly when pH data from the Buchanan group's studies were removed, pH explained 15% ($R^2$ = 0.1456) of the over- or under- prediction.

### *2.3.4.4 Effect of $a_w$*

The residuals of inactivation of *L. monocytogenes* for temperatures in the range of 0-45°C, normalised for the effect of temperature as per Section 2.3.4.3 were plotted against $a_w$ as shown in Figure 2.11. $A_w$ explained 6% ($R^2$ = 0.0598) of the over- or under-prediction of the simple Arrhenius model. When the non-refereed datasets were removed from the 0-45°C plot shown in Figure 2.11, $a_w$ explained 8% ($R^2$ = 0.0802) of the over- or under-prediction of the Arrhenius model. When $a_w$ data from the Buchanan group's studies were also removed, however, $a_w$ explained over 50% ($R^2$ = 0.5042) of the over- or under- prediction.

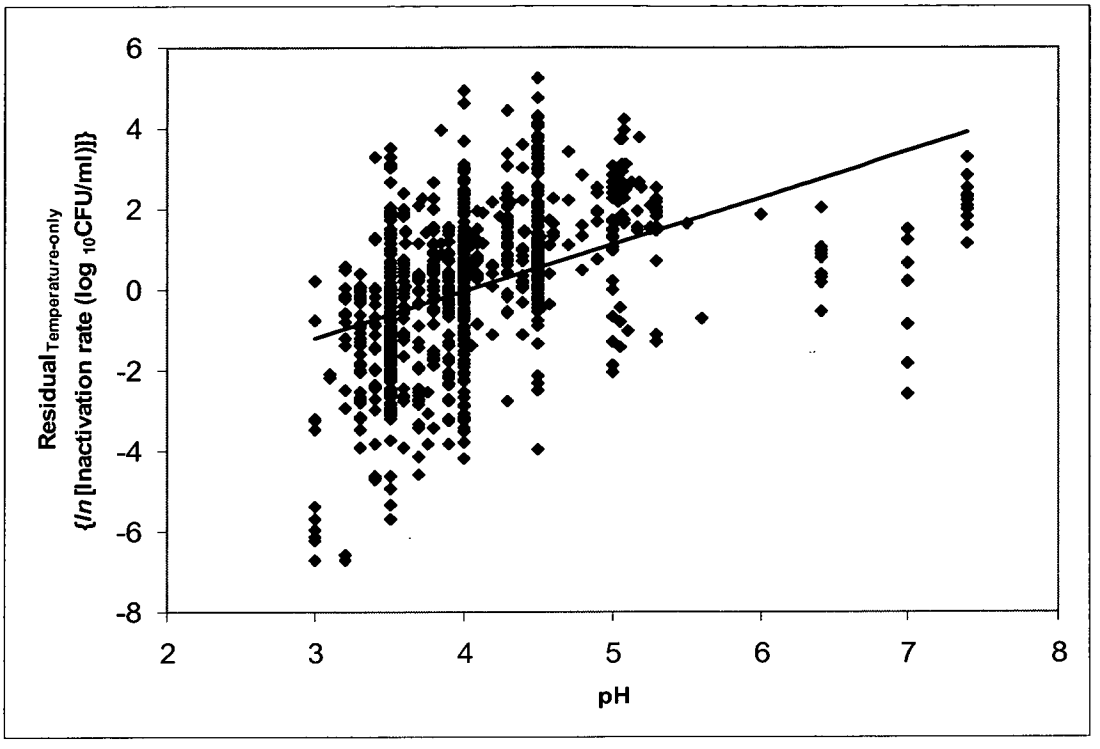


**Figure 2.10.** Residuals of *ln*(inactivation rate) of *L. monocytogenes* , after normalisation for the predicted effect of temperature (in the range 0 – 42°C) , *vs.* the pH of the treatment for measurements . The regression equation fitted to the data is $y = 1.1659x - 4.7064$ ($R^2 = 0.2142$)

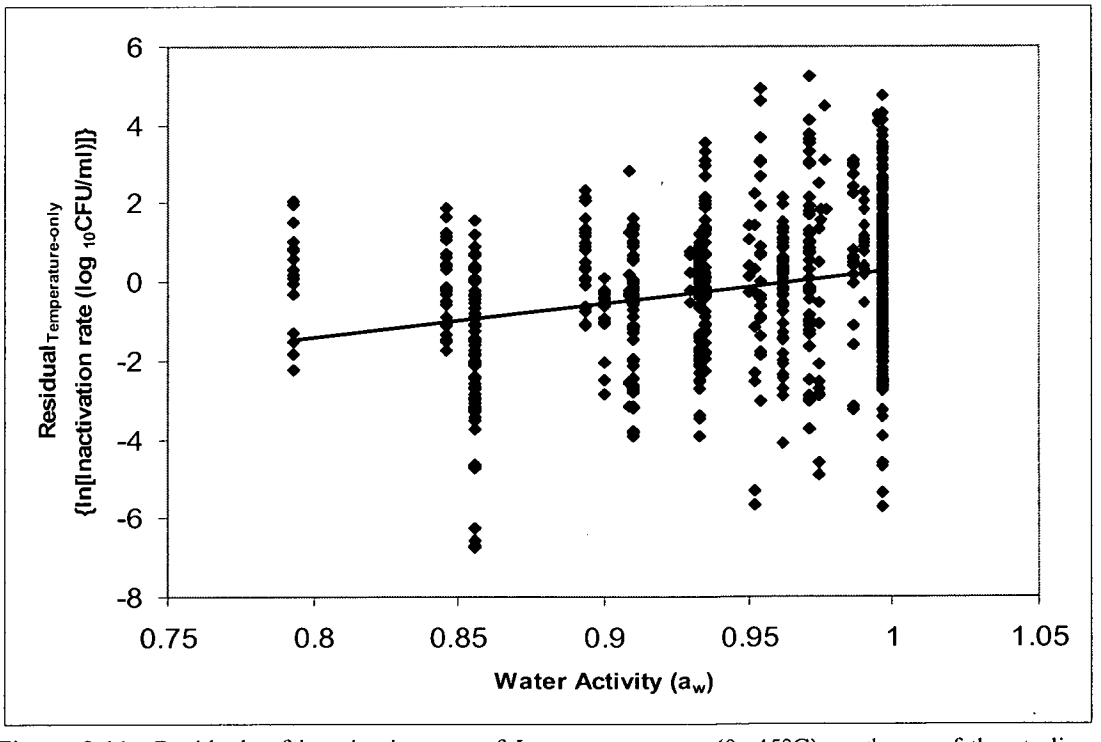


**Figure 2.11.** Residuals of inactivation rate of *L. monocytogenes* (0- 45°C) *vs.* the $a_w$ of the studies. The regression equation fitted to the data is $y = 8.4845x - 8.1905$ ($R^2 = 0.0598$).

## 2.4 Discussion

Previous studies (Ross and Shadbolt 2001; McQuestin et al. 2006; McQuestin et al. 2009) have found that non-thermal temperatures strongly influence the rate of *E. coli* inactivation in fermented meat products when sufficient non-thermal hurdles are in place to prevent its growth. Fermented meat products are characterised by low pH and $a_w$, high levels of lactic acid and remain uncooked during their fermentation and maturation. Zhang et al (2010) investigated the effect of non-lethal temperature on the inactivation rates of an altogether different bacterial species, *L. moncytogenes*. Those authors found similarities between the effects of non-thermal temperatures on the inactivation of both *E. coli* and *L. monocytogenes* when pH and $a_w$ were growth limiting, but constant. The work documented in this chapter was initiated to test further the generality of this relationship between non-lethal temperature and the rate of inactivation of *L. monocytogenes* in systems that mimic semi-hard cheeses, which like salami, are fermented, uncooked food products with low pH and low $a_w$ and contain high levels of lactic acid starter cultures. Lactic acid was the preferred acidulant for the experiments presented in this chapter, however, attempts to reduce the pH of TSB to 3.5 using lactic acid during pilot studies were not effective due to the buffering capacity of the broth and resulted in the formation of precipitate. HCl was able to reduce the pH of TSB to 3.5 without adversely affecting the $a_w$ or forming a precipitate. Moreover, it is difficult to separate the effects of organic acids and pH, because lower pH accentuates the effect of the acid due to association of protons with the anion, making it more lipophilic. Accordingly, a reductionist approach can be informative. It is also recognised that organic acids and mineral acids may induce different responses from bacteria (see Section 5.4).

The results of this work show that the rate of inactivation of *L. monocytogenes* in broth acidified to pH 3.5 with $a_w$ of 0.90, and in the temperature range of 5-45°C, is strongly affected by temperature, despite variability between strains. These broth studies can be directly related to the inactivation rates of *L. monocytogenes* presented by Zhang et al (2010) as the studies were undertaken in cooperation so as to generate complementary data. Zhang et al (2010) found no systematic differences between the mean inactivation rates of *L. monocytogenes* and *E. coli* but examination of the inactivation kinetics of *L. monocytogenes* at 45°C showed marked differences to

those of *E. coli* at the same temperature. This difference in response at 45°C was also observed in the studies described here as shown in Figure 2.2d. The inactivation kinetics of *L. monocytogenes* at 45°C, in both the current work and that described by Zhang et al (2010), are characterised initially by a rapid rate of inactivation followed by a decline in inactivation rate over time, yet this pattern is not evident at the other temperatures tested. The biphasic, or curvilinear, inactivation kinetics of *L. monocytogenes* at 45°C may be explained by the fact that this temperature is at, or near, the upper temperature limit of growth for the species (Ross et al. 2000) and incubation at that temperature *per se* may have been lethal to *L. moncytogenes*. When the 45°C data were removed from the Arrhenius model (Figure 2.3) the effect of temperature on the inactivation of *L. monocytogenes* increased from 84% to 88% and the RMSE was reduced from 0.718 to 0.298.

In Figure 2.1 together with Figures B.1 and B.2 (in Appendix B), the inactivation rates of *L. monocytogenes* at 5°C appeared to be anomalously fast when compared with the rates at 10°C. This is also clear when those rates are compared with the rates of inactivation for *L. monocytogenes* at the same temperature reported by Zhang et al (2010). The mean inactivation rate for the three strains used in this study is 0.027 and the mean inactivation rates for the three strains (with only one common strain. ATCC 19115) used by Zhang et al (2010) is 0.0046. At 5°C the inactivation rate for the common strain ATCC 19115 in the current work was 0.0274 compared to 0.0055 in the results from Zhang et al (2010). It is not clear why the inactivation rates at 5°C were anomalously fast. The data included in this study are from a second study to quantify inactivation rates for *L. monocytogenes* at this temperature. In the earlier study, a number of technical issues were encountered with the refrigeration units of the water baths used as well as the shaking mechanisms, causing inaccurate temperature readings. Inactivation rate determined from those initial studies were faster than the ones presented in thesis. It is possible that the issues with holding temperature that were evident in the first experiment were not completely rectified on the second attempt.

A further ten inactivation kinetic studies of *L. monocytogenes* were undertaken (Figures 2.4 and 2.5) using the original three strains supplemented with strains Scott A and FW035-0035 which were studied by Zhang et al (2010). In these

supplementary studies the temperature was constant at 15°C, pH was increased to 4.5 and two $a_w$ levels (0.93 and 0.95) were tested. Using a predictive model based on Tienungoon et al (2000), these levels of the environmental parameters were chosen because they are at, or near to, the minimal combinations of pH and $a_w$ that would *prevent* the growth of *L. monocytogenes* at 15°C, i.e., near to the mildest possible growth preventing conditions. Under those conditions, neither the pH nor the $a_w$ alone are predicted to prevent growth. The pH and $a_w$ for the extra inactivations are all less extreme than the original conditions and the inactivation rates are slower than in the original inactivations. This is also supported by the inactivation rates presented in Table 2.3 where all inactivation rates at $a_w$ 0.95 are slower than at 0.93. At 15°C, pH 4.5, $a_w$ 0.95 is predicted to allow growth for some strains, but not others, according to Tienungoon et al (2000) while $a_w$ 0.93 is predicted to prevent the growth of most strains. The observations of Tienungoon et al (2000) are borne out by the results in Figures 2.4 and 2.5. The two strains introduced at this stage appear to inactivating more slowly than that the three strains originally studied at $a_w$ 0.93. Strain Scott A exhibits slow inactivation at $a_w$ 0.93 but strain FW035-0035 shows initial decreases in population numbers before slowing at approximately 350 hours after which inactivation appears to have ceased, at least up until the cessation of the experiment at around 600 hours. The strain is known to be acid resistant (Bowman 2009). Analysis of the inactivation rates for the additional experiments with the original results at 15°C (Table 2.3) indicates that the rates of inactivation for the two new strains were significantly different to all of the inactivation rates at 15°C for the original three strains regardless of the $a_w$ or pH. Due to this the inactivation rates for the two additional strains were not used in any further analyses. Removal of those rates affected the goodness-of-fit of the simple Arrhenius model with the $R^2$ increasing from 0.77 (see Figure 2.6) to 0.83. With the removal of the 45 and 5°C data the $R^2$ increased to 0.87. *ln*(inactivation rate) responses from 10 to 35°C are very well explained by the Arrhenius model which agrees with the observations of McQuestin (2006) and Zhang et al (2010). With the 5°C data included, the inactivation rate data in Figure 2.6 appear curvilinear for the effect of temperature and another model was investigated to see whether it could better describe the effect of temperature on inactivation. A quadratic line was fitted to the Arrhenius-transformed data (without 45°C) and showed a significantly improved fit to the data compared to simple Arrhenius model (i.e. straight line) and explained 89% of the

variance in the transformed inactivation data. When the same analysis was undertaken without the 5°C data the fit of the quadratic line was not significantly better than the Arrhenius model. Thus if the 5°C data are truly anomalous then the linear Arrhenius model accurately predicts the effect of non-thermal inactivation for *L. moncytogenes*. A further discussion about low temperature anomalies follows.

Figure 2.7 compares the Arrhenius models derived from a number of studies (McQuestin et al. 2006; McQuestin et al. 2009; Zhang et al. 2010) with the model developed in this study from *L. monocytogenes* in inimical broth. Data from Zhang et al (2010) were derived from studies of the inactivation of *L. monocytogenes* and *E. coli* in broth at pH 3.5 and aw 0.90. Data from McQuestin et al (2009) were derived from a collation of published studies of *E. coli* inactivation in a variety of fermented meat products and analogous aqueous systems, while data from McQuestin (2006) were derived from novel studies of *E. coli* inactivation in salami and an analogous broth system. The slope of the Arrhenius plots appear to be consistent for the model developed for *L. monocytogenes* in this study and all the other models for *L. monocytogenes* and *E. coli*. Statistical analysis of the slopes of the Arrhenius models for the current study and those for *E. coli* and *L. monocytogenes* from Zhang et al (2010) was undertaken. Both models for the inactivation of *L. monocytogenes* were analysed using the reduced data sets (no 45°C data) for the reasons stated above. The slopes of the two models were significantly different until the 5°C data from the current study were removed. Zhang et al (2010) found no significant difference between the rates of inactivation for *E. coli* and *L. monocytogenes* at non-thermal temperature ranges under inimical broth conditions. The results from this work and the comparison with the study by Zhang et al (2010) further suggests that the inactivation of both bacterial species under inimical broth conditions is equally influenced by non-lethal temperature. The slopes of all of the modelled lines in Figure 2.7 show the relative effects of temperature and suggest that the relative effects of non-thermal temperature on bacterial inactivation do not differ systematically between the species studied nor the inimical environment, despite there being systematic differences in the observed *absolute* inactivation rates in studies using fermented meats and those using broths. Inactivation rates in foods appear to be slower than in broths as noted in McQuestin et al (2006) but the mechanism of the difference remains unresolved.

The $R^2$ values for all the models compared in Figure 2.7 are ≥ 0.60 indicating that temperature on its own accounts for at least 60% of the variability in the observed *ln* inactivation data despite relatively large differences in pH and water activities in some of the studies, as well as a diversity of strains. However, it is also apparent from the $R^2$ values that other factors also influence the observed rate of inactivation. In the controlled broth study reported here, the only independent variables other than temperature were bacterial strain and "experimental error". These variables were found to contribute little to the variability in response, with the effect of temperature explaining 84 to 88% of the variability in the *ln*(inactivation rate) data for *L. monocytogenes* when exposed to pH 3.5 and $a_w$ 0.90 at a range of non-lethal temperatures. This was also found by Zhang et al (2010) except that the independent variables of bacterial strain and bacterial species were not important predictors of the variability of the inactivation response. In that study temperature explained over 90% of the observed variability in the *ln*(inactivation rate) data when multiple strains of *L. monocytogenes* and *E. coli* were exposed to pH 3.5 and aw 0.90. The current study supports the hypothesis proposed by Ross et al (2008), McQuestin et al (2009), and Zhang et al (2010), that non-thermal bacterial inactivation rates in inimical environments are governed primarily by temperature.

The studies analysed in the collation of literature data (Figure 2.8) are based on combinations of pH and $a_w$, that alone or in combination, preclude bacterial growth and would be expected, under the hypothesis of Ross et al. (2009), to cause the inactivation of *L. monocytogenes* in food and broth systems. Multiple studies included in this meta-analysis reported that increased temperature increases the non-thermal inactivation rate of *L. monocytogenes* in various foods and broths (Shahamat et al. 1980; Ahamad and Marth 1989; El-Shenawy and Marth 1990; Larson et al. 1999). Similarly, multiple studies included in the meta-analysis of *E. coli* inactivation in fermented meats by McQuestin et al (2009) also reported such a correlation. The literature analysis reported by McQuestin et al (2009) for the inactivation of *E. coli* in fermented meats at temperatures of 0-47°C and for a range of pH, $a_w$, strains and product formulations, reported that temperature accounts for 60% of the variance in the inactivation rate data. The literature analysis reported here for the inactivation of *L. monocytogenes* in foods and aqueous broths at temperatures of 0-42° (Figure 2.9) found that temperature accounts for only 24% of

the observed variance in the inactivation data suggesting that other factors are more influential to the non-thermal inactivation of *L. monocytogenes* than for *E. coli.* The studies collated in this work included experiments in cheese, butters, mayonnaises, milk, coleslaw, artificial gravy, yogurt, fermented meats, orange juice, broths and broths with added sanitisers (Table 2.6). It is possible that the range of conditions used in the studies of this collation were more extreme than in the collation used by McQuestin et al (2009) which was restricted to salami-relevant studies.

Examination of individual datasets, which provided inactivation rates at three or more temperatures, highlighted a group of studies (FSA-IRF and FSA-CCFRA in Table 2.6) that did not show the same dominant effect of temperature as the data in this study, and data that will be presented in later chapters of this thesis. All of those studies were derived from the ComBase database and are from the same research group, and remain unpublished in the peer-reviewed literature. When the studies were removed from the analysis, the Arrhenius model had an improved $R^2$ of 0.2732. The data disaggregation also brought to light modelling datasets in the ComBase database from papers by the Buchanan group (Buchanan and Phillips 1989; Buchanan et al. 1994; Buchanan and Golden 1995; Buchanan et al. 1997) that, when evaluated as an Arrhenius model (data treated as one dataset), had a positive slope (i.e. increasing temperature predicted to decrease inactivation rate) and an $R^2$ of 0.08. Removal of the Buchanan datasets from refereed literature data only, increased the $R^2$ of the Arrhenius model to 0.4746, i.e. 47% of the variability explained by temperature. The Buchanan group datasets include modelled, rather than raw data, which may explain the discrepancy in the relationship between non-thermal inactivation and temperature seen in the modelled studies when compared to raw datasets. In examining the relationship between non-lethal temperature and the inactivation of pathogenic bacteria in foods, it is preferable to use raw data rather than modelled data.

Datasets were also differentiated according to the medium used for the challenge studies. Inactivation data derived from aqueous broth systems were found to have an $R^2$ of 0.1937. This differs from the observations of inactivation kinetics of *L. monocytogenes* in broth in this study and those from Zhang et al (2010) and McQuestin et al (2009) for the inactivation of *L. monocytogenes* and *E. coli* in

aqueous broth systems, and as shown in Figure 2.7. In those studies $R^2$ values were above 0.60. Inactivation data derived from foods showed that temperature accounts for 42% ($R^2 = 0.4175$) of the variance in those observed *ln*(inactivation rate) data for *L. monocytogenes* and is consistent with the observations of McQuestin et al (2009) for the inactivation of *E. coli* in fermented meats.

Analysis of the inactivation data from the literature for *L. monocytogenes* suggests that more factors influence its inactivation rate at non-lethal temperature than reported for *E. coli* by McQuestin et al (2009). The relationship between inactivation rate and pH or $a_w$ was examined for the studies collated here, with pH accounting for 21% and $a_w$ accounting for only 6% of the over- or under- prediction of the Arrhenius model. The influence of these parameters changed, however, with the removal of specific data sets, including non-refereed data and modelled data. pH accounted for 25% of the over- or under- prediction of the model when the non-refereed datasets were removed and 15% when the modelled data were also removed. Water activity explained only 8% of the variation of the Arrhenius model when the FSA non-refereed datasets were removed. Removal of $a_w$ data derived from the modelling studies, however, increased the $R^2$ to 0.5042 suggesting that $a_w$ explained 50% of the over- or under- prediction of the Arrhenius model. This apparently strong influence may be because, once the FSA and Buchanan datasets were removed, few of the remaining studies contained $a_w$ data. Thirty eight individual studies had very low $a_w$ (below 0.75) and formed part of the FSA datasets on ComBase. When they were included in the original Arrhenius model in Figure 2.7 (data not shown) the $R^2$ was 0.2479. Those datasets were excluded from the final meta-analysis because of the very low $a_w$ values. Specifically, as $a_w$ decreases in a system, it begins ultimately to act as a preservative for food, or bacteria, due to suppression of the chemical activity of water in those environments, a process sometimes referred to as the food or organism entering a 'glassy' state (Aguilera and Karel 1997). This suggests that the processes of inactivation are mediated *via* chemical reactions occurring, at least in part in the aqueous phase. The pH and $a_w$ data used were also separated into studies undertaken in broth and foods (data not shown) in order to investigate whether a matrix effect was apparent. Disagregation of the data used in Figures 2.10 and 2.11 (food *vs.* broth) indicated that out of those 1100 data points only 209 were obtained from food products. Of those, only 31

included $a_w$ readings and only 177 included pH readings. It was, therefore, concluded that adequate analysis could not be undertaken with the limited data .

The analysis of non-thermal inactivation rates for *L. monocytogenes* from the literature and ComBase did not show as strong a dominance of temperature as indicated by meta-analysis of non-thermal inactivation of *E. coli* presented by McQuestin et al (2009). A much larger range of food types were included in the analysis of *L. monocytogenes* inactivation including complex and non-homogenous foods such as cheeses, butters, mayonnaises, yogurts, and simulated foods as well as fermented meats with many different processing conditions and ingredients. Many of these foods, such as those that are water in oil emulsions (butter and cheese), have different local and global properties to which bacteria are exposed. Some properties of these foods potentially could provide a protective effect for vegetative bacteria and slow the rate of inactivation. The large number of strains used in the individual studies may have also affected the rates of inactivation. Strain dependent behaviour in *L. monocytogenes* was reported by Faleiro et al (2003). Differences in strain behaviour for *E. coli* were also identified by McQuestin et al (2009) and in the work presented here in Section 2.3.2. Strain variation can be a very important factor in analyses such as those undertaken in this work. For example, the differences in the mean inactivation rates of *L. monocytogenes* strains Scott A and FW035-0035 are equivalent to approximately a 10°C difference in temperature compared to predictions from the fitted line in Figure 2.6

Finally the data presented in the meta-analysis in Figure 2.9 together with the broth inactivation data in Figure 2.6 show a curvilinear response to temperature. Initially it was postulated that the meta-analysis data was skewed by low temperature data and concluded that the data may be biased for some reason. For example, collection of inactivation rate data at low temperatures (very slow inactivation rates) could be biased since investigators are less likely to observe and record results for very slow experiments due to time constraints. Arguing against this idea, however, is that a similar effect is seen in the data generated in this study. The observations of Ross et al (2008), McQuestin et al (2009), and Zhang et al (2010), that non-thermal bacterial inactivation rates in inimical environments are governed primarily by temperature regardless of bacterial species or inimical environment may not be correct. A

curvilinear model explained the effect of temperature on the data used in the meta-analysis (data not shown) and the broth inactivations in this study better than a linear model, i.e. the Arrhenius model may be inadequate to describe the non-thermal inactivation of *L. moncytogenes.* Further work is required to compare the data sets from McQuestin et al (2009) and the meta-analysis in this study, firstly test whether the effects of temperature as described by the linear models are significantly different between the species and secondly to test if the data from the *E. coli* meta-analysis is also significantly better described by a quadratic, rather than a linear, model. Access to the raw data from McQuestin et al (2009), however, was not readily available at the time of writing.

## 2.5 Conclusion

This study used a broth model to investigate the inactivation kinetics of *L. monocytogenes* in response to non-thermal temperature in inimical conditions and to test the hypothesis that temperature is the dominant predictor of non-thermal inactivation of bacteria in inimical environments, regardless of the environment, bacterial species etc. The rate of inactivation of *E. coli* and *L. monocytogenes* in aqueous broth systems and fermented meats, in which pH and aw, in combination or alone, preclude growth has been shown to be strongly dependent on non-lethal temperature (Ross and Shadbolt 2001; Ross et al. 2008; McQuestin et al. 2009; Zhang et al. 2010). The results from this novel study support the hypothesis that temperature is the dominant factor affecting the inactivation of vegetative bacteria when they are prevented from growth by inimical conditions and highlight the usefulness of considering the effect of processing and maturation temperature in the production of foods, such as cheese and salami, that undergo minimal thermal processing. However, while the relative effect appears to be true, the absolute rates of inactivation do seem to vary, in contradiction to the suggestion of Zhang et al (2010).

The effect of temperature on the inactivation rates of *L. monocytogenes* found in the literature and in databases, however, was not as distinct as that found by McQuestin et al (2009) for the inactivation rates of *E. coli* in fermented meats and analogous broth systems. The range of food types used in challenge studies was more complex,

and the inclusion of modelled data and strain effects may have contributed to the variability in the in the inactivation rate data. Another factor is the relatively small number of challenge studies undertaken in foods at multiple temperatures.

# Chapter 3 Inactivation of Pathogenic Bacteria during Production of Raw and Pasteurised Semi-hard Cheese

## 3.1 Introduction

During the past decade in Australia, there has been lobbying and increasing demand by cheese makers and cheese enthusiasts to allow the production and sale of a wider array of raw milk cheeses than is currently permitted by the Australian Food Standards Code (FSANZ 2009). Arguments presented to support these demands include commonly held views of epicureans, importers, food writers, restaurateurs, specialty cheese makers and general consumers that cheeses made from raw milk have superior flavour and health properties than cheeses made from pasteurised milk. Pasteurisation of milk prior to cheese making is thought, by these proponents, to contribute to flavour loss due to biological and chemical changes caused by heat treatment, to retard the ripening process, and is claimed to have deleterious effects on the purported health benefits of raw milk cheese. Many also argue that whilst pasteurisation is an effective kill step it does not protect against post-production contamination or general bad production practice.

Resistance to the "push" to allow the domestic production of raw milk cheeses has its roots in the conservative Australian commercial dairy industry, which is geared for pasteurised cheese production on a large scale (Anonymous 2009). There is also a public perception in Australia that heat treated cheeses are a safe food and there exists strong public confidence since there have been no notable food safety incidents in Australia due to pasteurised cheese (http://www.ozfoodnet.org.au/). The heat treatment of milk and milk products is mandated via the Australian Food Standard Code (FSC) as an important public health measure to destroy most microbiological hazards that may be present in milk and has provided the benchmark public health and safety measure for dairy foods in Australia (FSANZ 2009). The FSC requires that cheese must be made from milk that has undergone a defined heat treatment prior to manufacture. Heat treatments may consist of the following:

- holding milk at no less than 72°C for 15 seconds (or by using a time and temperature combination providing an equivalent level of bacterial reduction);

- or heating to no less than 62°C for 15 minutes or more and storing cheese for more than 90 days before sale (FSANZ 2009).

The FSC currently prohibits the production and sale of many cheeses made from non-heat treated or raw milk for reasons of protection of public health and allows the import and sale of some raw milk cheeses assessed as 'safe' under the principle of equivalence (Anonymous 2005). The principle of equivalence is the recognition that the same level of food safety and hygiene can be achieved by applying alternative hazard control measures such as animal health, general hygiene practices on farm and at during milking, strict requirements relating to raw milk quality, high temperature curd cooking steps, specific moisture contents and minimum storage periods for finished cheeses (FSANZ 2009). Equivalence of food safety measures is recognised in the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (http://www.wto.org/english/tratop_e/sps_e/spsagr_e.htm) agreed in 1994 and the complementary WTO Agreement on Technical Barriers to Trade (http://www.wto.org/english/tratop_e/tbt_e/tbt_e.htm). These agreements require member countries to ensure their food safety measures are objective, science-based and consistent. The raw milk cheese-making processes in the countries of origin are also heavily monitored to minimise and detect any potential contamination with pathogenic bacteria. All of these controls are considered by Australian food safety authorities to provide acceptable levels of protection of public health. Extra hard grating cheeses produced in Italy from raw milk and Swiss hard cheeses such as Emmental, Gruyere and Sbrinz are currently imported and sold in Australia since they have been assessed to have an equivalent level of safety as cheeses made from heat treated milk. Australia has also recently approved the importation of Roquefort cheese, which is a raw ewe's milk blue mould-ripened cheese originating in France (Anonymous 2005). The three raw milk Swiss cheeses are currently allowed for sale in Australia with specific permission for these cheeses expressed in Standard 2.5.4. In addition, the sale of raw milk extra hard grating cheeses is specifically permitted

through an exemption to the heat treatment requirements in Standard 1.6.2. The sale of raw ewe's milk Roquefort is permitted through a clause in Standard 4.2.4A (FSANZ 2009). All exempted cheeses must also comply with requirements Standard 1.6.1 – "Microbiological Limits for Food" and any applicable State and Territory requirements in relation to cheese production.

As noted in Chapter 1, many challenge studies have been conducted to investigate the behaviour of *L. monocytogenes* and *E. coli* during the manufacture and ripening of cheeses made with milk intentionally inoculated with these pathogens. Ryser and Marth (1987b), Back et al (1993) and Papageorgiou and Marth (1989b) demonstrated that *L. monocytogenes* survives during production of Camembert and Feta cheeses. The pathogen grew in Camembert (Ryser and Marth 1987b; Back et al. 1993) and growth has been shown to also occur in butter, cultured buttermilk and yogurt (Choi et al. 1988; Olsen et al. 1988). Griffiths (1989) concluded that some *L. monocytogenes* cells could survive the process of making raw milk cheeses and remain viable in cheese, even after the 60-day holding period mandated by current FDA regulations for raw-milk cheeses sold in United States of America, provided pathogen levels were in excess of 1x $10^2$ cfu/ml in milk destined for cheese making. Griffiths (1989) also reported *L. monocytogenes* survived for up to twelve weeks in non-fat dry milk at room temperature and ten weeks in butter stored at 4-6 and 13°C.

Genigeorgis et al (1991) found that, of 24 types of cheeses surface inoculated with 3.95 – 4.36 log cells of *L. monocytogenes* after opening to mimic contamination of cheese from other raw foods after opening, soft cheeses (such as cottage, Queso Fresco, Brie, Telemes and Camembert) supported growth with average population increase of 1.4 log10. Growth was seen even at 4°C in cheese made without starter cultures (Genigeorgis et al. 1991). A number of cheeses made with starter cultures, at pH 4.9-5.5 and stored at temperatures 4, 8 and 30°C, did not support the growth and caused gradual inactivation of *L. moncytogenes*. The rate of inactivation for pathogens was higher at 30°C than the other storage temperatures. These data are not included in the meta-analysis in Chapter 2 because the data available were insufficient to determine inactivation rates. That the rate of inactivation was greatest at higher temperatures agrees with the observations of Zhang et al (2010) and McQuestin et al (2009), and many other studies (see Chapter 2) that increasing

temperature within the non-lethal range increases the rate of bacterial inactivation in inimical environments.

A number of manufacturing process parameters such as: the high curd cooking temperatures (53 to 56°C for up to 85 minutes), moulding the cheese and holding at 52 to 56°C for up to ten hours at pH 5.0, brine salting to reduce $a_w$ to 0.90 and extended ripening periods of 9 to 12 months duration, contribute to the microbiological safety of Italian hard cheeses Grana Padano and Parmigiano Reggiano made with raw milk (Pellegrino and Resmini 2001). Yousef and Marth (1990) found *L. monocytogenes* declined rapidly to undetectable levels in Parmesan cheese within 14-112 days, depending on the strain studied.

Ryser and Marth (1987a) found *L. monocytogenes* in Cheddar cheese ripened at 6 or 13°C and at pH 5.0 survived for more than 434 days. *L. monocytogenes* also survived for more that 115 days in Colby cheese at pH 5.0, up to 120 days in Blue cheese at pH 6.2 and for more than 28 days in cottage cheese (Ryser et al. 1985; Yousef and Marth 1988; Papageorgiou and Marth 1989a). Reitsma and Henning (1996) found *E. coli* decreased during the manufacture and ripening of Cheddar cheese, however, viable cells were still detected after 158 days of ripening. Schlesser et al (2006) found acid resistant *E. coli* O157:H7 survived longer than the 60-day withholding period for raw milk cheese in Cheddar made from raw milk inoculated with *E. coli*. All of the studies mentioned used ripening temperatures that correspond to traditional practices in commercial cheese production.

Larson et al (1999) determined the survival of *L. monocytogenes* in commercial cheese brines (up to 22% NaCl) collected from cheese factories in Wisconsin and northern Illinois in USA. Survival of *L. monocytogenes* inoculated into commercial cheese brines ranged from <7 days to over 259 days but did not correlate with pH, salt content, nitrogen content, mineral content, or inherent microbial populations but was negatively associated with addition of sodium hypochlorite at the dairy plant. The pathogen generally survived longer in brines held at 4°C than at 12°C.

The factors affecting survival and growth of pathogens in cheese are complex. Production of acid and inhibitory substances by starter cultures, and low water

activity, are some of the many factors that may negatively affect the survival and growth of pathogens in cheese. The likelihood that a pathogen will grow and survive in a cheese will vary with the type of cheese made (Eyles 1992). Hard cheeses are a harsh environment for pathogens and there is a relatively low risk that pathogens will survive in them. Soft ripened cheeses present a relatively high risk due to their higher pH and moisture levels and lower salting levels (Eyles 1992). Aged cheeses made from raw milk have a good safety record and a recent review by Donnelly (2001) found that hard, aged cheeses made from raw milk are microbiologically safe due to steps in their manufacture which "insure inactivation of microbial pathogens, or because of inherent parameters which inhibit microbial growth".

A review by Johnson et al (1990b) of epidemiological literature during the period 1948-1988 identified only six outbreaks of cheese-transmitted illness in USA during that forty year period. The most frequent causative factor in the outbreaks was post-pasteurisation contamination. No outbreaks were linked to Italian extra hard grating cheeses (Parmesan, Romano, and Provolone), and Swiss and Cheddar cheeses were rarely linked to outbreaks. Johnson et al (1990b) cited milk quality and management, lactic acid bacteria starter culture management, pH, salt, aging conditions and natural inhibitory compounds in raw milk rather than pasteurisation as contributing to raw cheese safety. The infrequency of large cheese-associated outbreaks during the period 1973-1992, as reported to the CDC, was noted in a review by Alterkruse et al (1998). Out of 32 cheese-associated outbreaks, only one was associated with Cheddar and five were linked to the consumption of soft Mexican style cheese (Alterkruse et al. 1998). None of the reported outbreaks was associated with raw-milk cheese that had been aged for more than 60 days.

A long-lasting outbreak of listeriosis with 122 cases during 1983 to 1987 in Vaud, Switzerland was linked to the consumption of Vacherin Mont D'Or soft cheese and had a mortality rate of 28% (Billie et al. 1992). The pathogen has also been responsible for two outbreaks of listeriosis in the US caused by consumption of Mexican soft cheeses made with combinations of raw and pasteurised milk. The first outbreak in 1985 in California had a mortality rate of 39% among 142 cases (Linnan et al. 1988). The plant that made the cheese was found to have *L. monocytogenes* as a common environmental contaminant. The second outbreak was caused by

consumption of homemade fresh Mexican style cheese in North Carolina in 2000 to 2001 that was sold by door-to-door vendors (Boggs et al. 2001).

Data from epidemiological surveys and from challenge studies indicate that soft cheeses provide favourable conditions for the growth and survival of *L. monocytogenes* and *E. coli* but hard dry cheeses do not. Semi-hard cheeses, such as short ripened Cheddar or Colby and Manchego, fall in between these two categories and have shown mixed results for the survival of pathogens. This study was undertaken to evaluate the behaviour of *L. monocytogenes* and *E. coli* when present as either contaminants in raw milk, or as post-pasteurisation contaminants in milk, bound for cheese making. With assistance from a professional cheese-maker, batches of raw and pasteurised semi-hard Manchego style cow's milk cheeses were prepared at the microbiology laboratories of the School of Agricultural Science, University of Tasmania. Milk was inoculated with either *L. monocytogenes* Scott A strain or *E. coli* M23 (a non-pathogenic strain) in both stationary and exponential stages of growth. Cheeses were prepared and stored at six temperatures in the range 4°C to 25°C. After inoculation with the challenge strains and at various stages during processing of the cheese and subsequent storage, *L. monocytogenes* or *E. coli* remaining in the cheese were enumerated using selective agar media. Changes in population number were modelled using simple linear regression, where appropriate, and rates of changes for both species plotted as a function of temperature using Arrhenius plots. Potential differences due to the physiological state of the cells at the time of cheese making were also evaluated by comparing inactivation rates of *L. monocytogenes* and *E. coli* at either stationary or exponential phases of growth.

## 3.2 Materials and Methods

### 3.2.1 Bacterial Strains and Media

In this study one *L. monocytogenes* and one *E. coli* strain were used to determine inactivation kinetics in semi-hard Manchego-style cheese made from raw cows' milk. The challenge organisms were *L. monocytogenes* strain Scott A (ATCC15413, a known pathogenic type strain widely used in research) and *E. coli* strain M23 OR:H- (a well characterised, acid tolerant, and non-pathogenic strain). *E. coli* strain M23 OR:H- was found to respond to acid stress similarly to the virulent *E. coli* strain O157:H- (Brown et al. 1997). All strains were obtained from the School of Agricultural Science Culture Collection (University of Tasmania). Cultures were maintained in nutrient broth (Oxoid CM1, Adelaide, SA, Australia) and 30% glycerol (Sigma-Aldrich, Melbourne, VIC, Australia), at -80°C. Strains were recovered on Brain Heart Infusion agar (BHA; Oxoid CM225 to which 15g $l^{-1}$ agar was added) at 37°C for 24h. The starter culture used for cheese production, CHOOZIT™ MM100 LYO 25 DCU (Danisco, Denmark), is a freeze-dried concentrated mesophilic lactic acid bacteria (LAB) preparation. LAB were resuscitated by inoculation into 200 mL of sterile UHT processed milk in a 250 mL Erlenmeyer flask and incubated at 30 (±1.0) °C in a shaking water bath (Ratek Instruments, Boronia, VIC, Australia) for one hour. LAB inoculation rates were 0.332g freeze-dried culture/10L of vat milk.

### 3.2.2 Inactivation of *L. monocytogenes* and *E. coli* in raw or pasteurised semi-hard cheese curd at 4, 7, 10, 15, and 25ºC

#### *3.2.2.1 Preparation of Stationary Phase Populations of* L. monocytogenes *and* E. coli

To prepare experimental inocula, *L. monocytogenes* and *E. coli* cells were removed from the surface of thawed stock culture (stored at -80°C) using a sterilised inoculation loop, plated to BHA supplemented with 0.1% sodium pyruvate (P8574 Sigma-Aldrich, Melbourne, VIC, Australia) (BHAP) and incubated for 24 (±0.25) hours at 37 (±0.5) °C. Five colonies of each bacterial strain on BHAP were inoculated to 100 mL Tryptone Soya Broth (Oxoid, CM0131) containing 0.6% yeast extract (Oxoid, CM0019; TSB-Ye) in a 250 mL Erlenmeyer flask and incubated

statically at 37(±0.5) °C for 24 (±0.25) hours to achieve a viable count of approximately 9.0 log CFU.mL$^{-1}$.

One mL aliquots of stationary phase *E. coli* were transferred to a 15 mL sterile tube. Cells were pelleted by centrifugation at 5000 rpm for 10 minutes at room temperature in a Universal 16A centrifuge. The supernatant was poured off and one mL of raw milk was used to resuspend the cell pellet. The cell suspension was then added to the bulk milk. The same process was performed for *L. moncytogenes*. Milk used to manufacture raw or pasteurised cheese was inoculated with only one species of pathogen. Immediately following inoculation a 1 mL aliquot was withdrawn for enumeration by spread plating of appropriate dilutions on selective agar as detailed in Section 3.2.2.5.

#### *3.2.2.2 Preparation of Exponential Phase Populations of* L. monocytogenes *and* E. coli

Five colonies from a 24-hour plate culture of *L. monocytogenes* were inoculated into 250mL of pre-warmed TSB-Ye in a sterile 500 mL Schott bottle. The primary broth was incubated in shaking water bath at 34°C (±0.1) until just visibly turbid (assessed "by eye" and determined by previous studies to correspond to approximately $10^6$ cfu.mL$^{-1}$). This inoculum was left undiluted, and 1.0ml was added to each of four sterile Schott bottles containing 250mL of TSB-Ye to achieve a starting density of ~$10^3$ cfu.mL$^{-1}$. The secondary culture was then incubated for approximately 12 hours at 17°C to reduce the growth rate so as to limit the populations to $\leq 10^6$ cfu.mL$^{-1}$ after overnight incubation. The culture was then shifted to 33°C, to acclimate the cultures and establish logarithmic growth at the acclimation temperature of 33°C (±0.1), i.e. the temperature at which the cheese was to be prepared. The culture was then monitored until visibly turbid (determined 'by eye'), thus providing an exponentially growing culture of ~$10^6$ cfu.mL$^{-1}$. The four exponential cultures were transferred to sterile 250mL centrifuge tubes. Cells were pelleted by centrifugation at 4500 rpm for 10 minutes at 4°C in an Avanti-J-301 centrifuge (Beckman Coulter, Australia). The supernatant was decanted and one mL of raw milk was used to resuspend each cell pellet. The cell suspensions were then added to the bulk milk. The same process was performed for *E. coli*. Immediately

following inoculation a 1 mL aliquot was withdrawn for enumeration by spread plating appropriate dilutions on selective agar (see Section 3.2.2.5).

### *3.2.2.3 Preparation of Raw and Pasteurised Semi-Hard Cheese Curds Using Stationary Phase Populations*

Unpasteurised cow's milk was obtained within an hour of milking from John Bignell's Dairy, Bream Creek, Tasmania, stored at 2°C and used for cheese making within 12 hours of milking. Cheese was made from 10 litres of either raw or pasteurised cow's milk. Milk for cheese making was not mixed with any other raw milk. Cheeses were made at weekly intervals. An equal number of raw and pasteurised cheeses were produced. For each batch of cheese, milk was pasteurised by heating to 64 ° C (±1.0) for 30 minutes. Milk for raw milk cheese making received no heat treatment. The MM100 LAB starter culture (see Section 3.2.1) was placed into an Erlenmeyer flask with 200 mL of sterile UHT milk and incubated in a shaking water bath at 30 (±1.0) °C for 1 hour prior to being added to the bulk milk. Immediately prior to commencement of cheese-making, a 2 mL aliquot of the raw cow's milk was withdrawn from the raw milk for pH measurement using a model 250A pH meter (Orion Research Inc. USA) fitted with an Activon AEP 433 flat tip probe (Activon Scientific Products Co. Pty. Ltd., Australia). The pH meter was calibrated prior to use according to the manufacturer's instructions with standard pH solutions (pH 7.00 and pH 4.00). One mL samples of raw milk were also serially diluted and plated onto selective plates (see section 3.2.2.5 below for details of methods) to check for milk contaminants that may be able to grow on the selective plates. The milk was added to a 16 L sterile stainless steel saucepan suspended inside a larger 20 L saucepan filled with water and warmed on a hotplate. Once the raw milk achieved a temperature of 32°C (±1.0), starter cultures and challenge organism cultures were added to the milk. Non-animal rennet (Cheeselinks, Australia) was added at the rate of 2.5 mL per 10 L of milk and rennet-to-cut time was between 45 minutes and one hour.

The curd was cut into approximately 1.5 cm cubes and allowed to 'rest' for 5 minutes. The curd was then cooked with the temperature rising from 32°C to 38°C (±1.0) over a 40-minute period. The curd was also stirred continuously during the cook period. Curds were poured into one of three sterile cheese hoops in

approximately equal amounts. Whey was collected, disinfected (by boiling and addition of chlorine-based disinfectant) and discarded. Curd masses were turned back into the hoop at half hourly intervals over a 3 to 4 hour period. Hooped curds were then left at 20°C overnight before being placed into a saturated brine solution for 8 hours. Cheeses were turned over after 4 hours. Saturated cheese brine was made by placing enough NaCl (Ajax Finechem, Taren Point, NSW, Australia) to 5 litres of boiled water so that the NaCl no longer dissolved and settled at the bottom of the container. After brining, cheeses were left at 20°C overnight to dry. Cheeses were then cut, using a sterile stainless steel kitchen knife in a Class 2 biological laminar flow hood, into 10 g (±2.0) pieces. Cheese pieces were individually inserted into separate polyethylene shrink barrier bags (B471 end seal bag, Cryovac, Victoria, Australia) and vacuum packaged with a Technovac T60 vacuum packer (San Paolo, Italy) before being incubated at one of six experimental ripening temperatures (4, 7, 10, 15, 20 or 25 °C). Water activity ($a_w$) was measured after drying and before packaging using an Aqualab CX2 dew point instrument (Decagon Devices, Pullman Washington, USA). The pH was measured throughout the cheese manufacturing process and again when $a_w$ was measured.

#### *3.2.2.4 Preparation of Raw and Pasteurised Semi-Hard Cheese Curds Using Exponential Phase Populations*

One batch (using 10L of milk) of both the raw and pasteurised semi-hard cheese curds, inoculated with either exponential phase cultures of *L. monocytogenes* and *E. coli* was prepared as detailed in Section 3.2.2.2. Vacuum packaged cheese samples were incubated at the experimental ripening temperature of 15°C only.

#### *3.2.2.5 Sampling and Enumeration of Challenge Organisms*

Cheese was sampled as described in Section 7.3 of Australian Standard 1766.3.15-1994 (FSANZ 2009), i.e. Method 3.15 Examination of specific products-Cheese. Cheese samples were transferred to filter stomacher bags (LabServ) and mixed with 90mL (±18.0) of pre-warmed (40-45°C) 20g/L sodium citrate solution and 0.1% peptone solution. Samples were homogenised for 3 minutes at 20°C in a stomacher blender (Colworth Stomacher 400, AJ Seward, London, UK). On each sampling day, cheese samples from two individually vacuum-packed bags per treatment

temperature were analysed (duplicates from one biological replicate). Appropriate serial dilutions in 0.1% peptone solution were prepared.

*L. monocytogenes* was enumerated by surface plating either 50, 100 or 250 µL volumes of appropriate dilutions using an Autoplate 4000 spiral plater (Spiral Biotech Inc., Bethesda, MA, USA) onto PALCAM Agar (Oxoid CM0877 with SR0150 supplement) and Listeria Selective Agar (Oxford Formulation) (Oxoid CM0856 with SR0140 supplement) and counting colonies with morphology consistent with typical *L. monocytogenes* on these media. Plates were incubated for 48 hrs at 37 (±0.5) °C. *E. coli* was enumerated by plating appropriate dilutions onto Eosin Methylene Blue agar (Modified) Levine (Oxoid CM0069) and counting colonies with morphology consistent with typical *E. coli* on this medium. Plates were incubated for 24 hrs at 37 (±0.5) °C. All selective/differential media were prepared in accordance with the manufacturer's instructions. Due to the use of very high inoculation levels of challenge organisms in the cheeses biochemical confirmation of colonies growing upon PALCAM, Listeria Selective and EMB agar was not undertaken in this work as it was assumed that most, if not all, colonies counted arose frome from challenge organisms.

Numbers of challenge organisms at each sampling time were plotted as $Log_{10}CFU.g^{-1}$ vs. time. From these plots, inactivation rates were determined by linear regression of the $log_{10}$CFU-vs-time data using Microsoft ® Excel. Inactivation rates were then plotted on Arrhenius plots (*ln*(inactivation rate) *vs.* 1/temperature[K]) to determine the temperature dependence of inactivation rate. Significance of temperature, as a factor influencing inactivation rate, was estimated by calculating $R^2$ values for both *L. monocytogenes* and *E. coli* data sets. The inactivation rates for each organism at all temperatures were calculated from the data relevant to times only after the curd formed (for explanation see Section 3.3.1).

#### *3.2.2.6 Comparison of Arrhenius models*

The Arrhenius model generated for non-thermal inactivation of *L. monocytogenes* and *E. coli* in this study was compared with the novel Arrhenius models generated in

broths (described Chapter 2 for *L. moncytogenes*) and those generated by Zhang et al (2010) for *L. monocytogenes* and *E. coli* and McQuestin et al (2009) for *E. coli*.

## 3.3 Results

### 3.3.1 Inoculum Partitioning, pH and Water Activity ($a_w$)

Raw milk samples were plated onto selective agar prior to the commencement of each session of cheese making and at no time were milk contaminants found to grow on selective agar (data not shown). The first batch of semi-hard cheese produced was also used to determine what proportion of the introduced challenge organism, if any, was lost to the whey portion discarded during cheese making. The results are shown in Figure 3.1 and clearly show that the bulk of the inoculum stays in the curd.

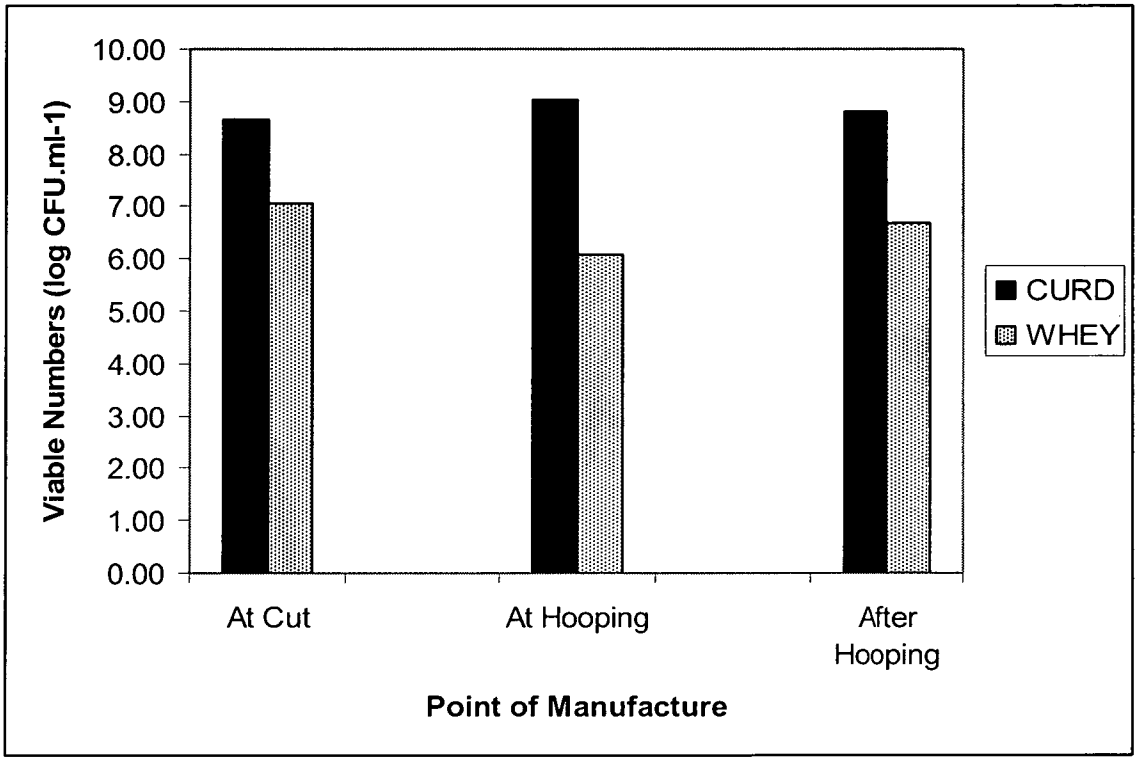


**Figure 3.1.** Histogram showing the difference in numbers of *L. monocytogenes* in the curd and whey fractions during the first 20 hours of manufacture of Manchego style cheese.

Figure 3.2 shows the evolution of pH in semi-hard cheese curds made from raw milk. The changes were very similar to those for cheese curds made from pasteurised milk. In each case the pH fell to below pH 5.0 within 24 hours of manufacture. The pH of the cheeses fell to 4.7 after 23 days of ripening (not shown in Figure 3.2), with cheeses inoculated with *L. monocytogenes* having a pH of 4.6 after 90 days of ripening (not shown in Figure 3.2). Before salting, $a_w$ levels were around 0.96 for all cheeses. $A_w$ was between 0.91 - 0.92 once the brine-salted cheese had dried and was

prepared for vacuum packaging. The $a_w$ of the vacuum packaged cheese was periodically determined throughout the experiments but did not change from that measured when the pieces were packaged.

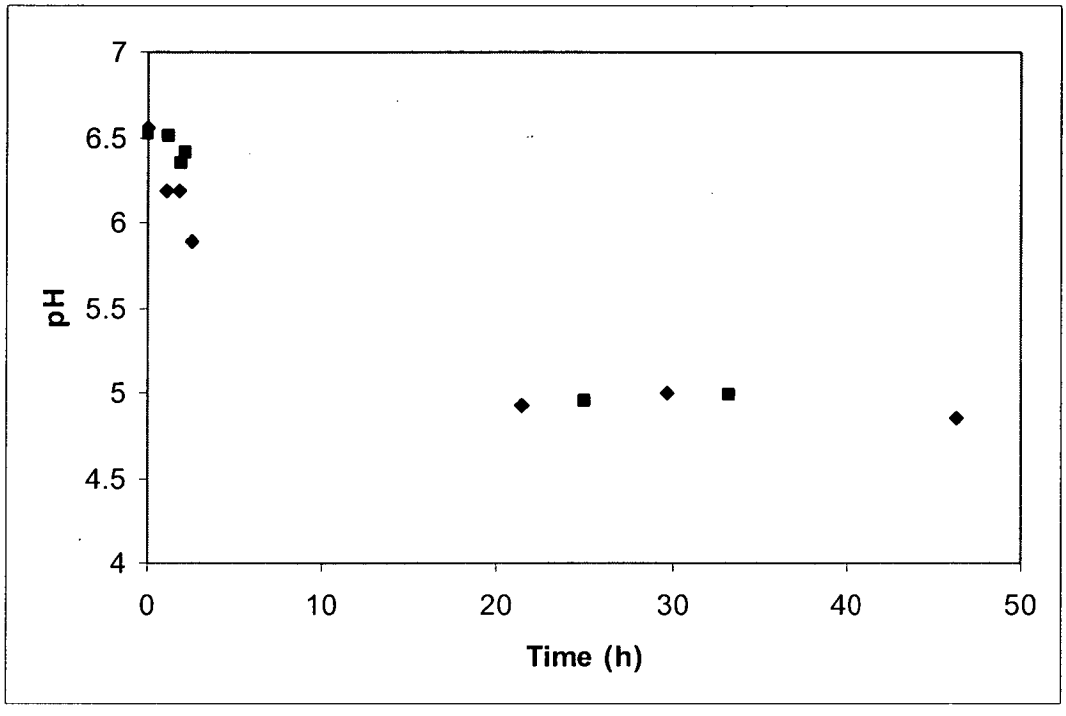


**Figure 3.2** The change in pH in semi-hard cheeses made from raw milk containing *L. monocytogenes* (♦) and *E. coli* (■).

### 3.3.2 Inactivation of Stationary Phase *L. monocytogenes* and *E. coli* populations in Raw and Pasteurised Semi-Hard Cheese Curds at 4, 7, 10, 15, and 25ºC

Inactivation curves for *E. coli* and *L. monocytogenes* at 4°C, 7°C, 10°C, 15°C and 25°C in both pasteurised and raw milk semi-hard cheeses are shown in Figure 3.3. There is variability between individual datasets, however, the data at all temperatures indicates that the both bacterial species are inactivated in semi-hard cheese. The effect of increasing temperature is also consistent in both raw and pasteurised cheeses and between species with more rapid inactivation occurring at 25°C when compared with 4°C. The data is Figure 3.3 also suggest that the inactivation rate of *L. monocytogenes* in semi-hard cheese is slower than that of *E. coli*. The inactivation kinetics of *L. monocytogenes* in raw milk cheese also appear to be biphasic at 4°C, 7°C, and 10°C.

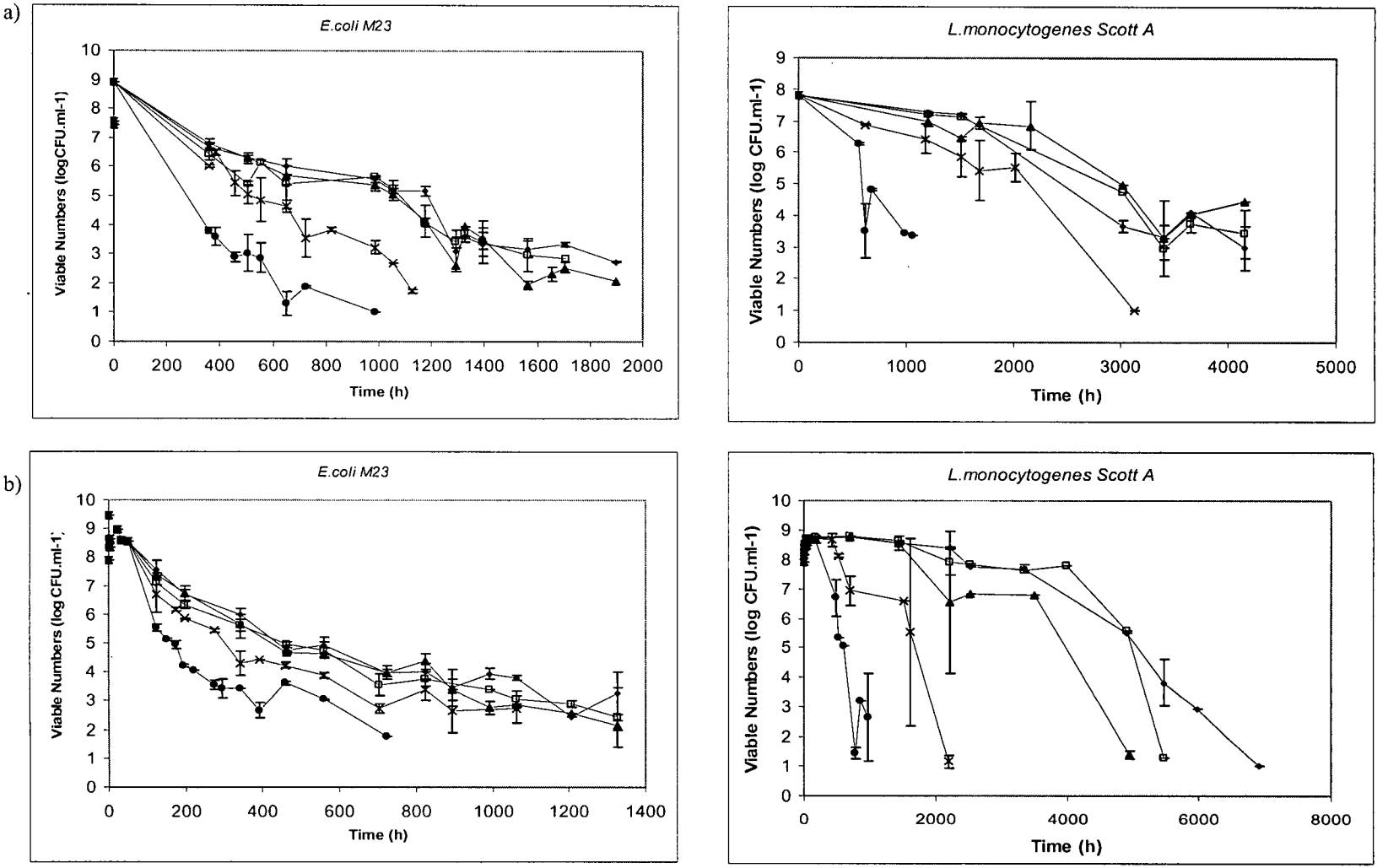


**Figure 3.3.** Non-thermal inactivation curves for *E. coli* M23 and *L. monocytogenes* Scott A at 4°C (♦), 7°C (□), 10°C (▲), 15°C (×) and 25°C (•) in (a) Pasteurised milk semi-hard cheese and (b) Raw milk semi-hard cheese.

Table 3.1 shows the inactivation rates calculated by simple linear regression from the inactivation curves for each species at all temperatures. Only data relevant to times after curd formation (approximately one hour after inoculation and renneting) were used. The inactivation rates in Table 3.1 were obtained by fitting a linear model to the curves in Figure 3.3; however, it is apparent that an initial increase in cell numbers occurred over the first few hours of processing. This corresponds with curd formation and removal of the whey and is likely to result from concentration of the cells into the curd, rather than being due to microbial growth. Apart from this apparent increase, in no experiment and at none of the temperatures considered, was growth of either organism observed. A linear model was also applied to the steepest parts of the inactivation curves for *L. monocytogenes* in raw milk cheese at 4°C, 7°C, and 10°C and these are presented in Table 3.2. The steepest part of the curve was used in this instance since population rates for *L. monocytogenes* in raw milk cheese remained steady (after the initial population increased caused by curd entrapment) until 700 hours when numbers began to decline. The linear model was applied arbitrarily to the inactivation curves from the same time point for each temperature (the first sample taken after 700 hours).

**Table 3.1.** Rates of inactivation of *L. monocytogenes* and *E. coli* in raw and pasteurised semi-hard cheese at various temperatures.

| *Temp. (°C)* | *E. coli Raw Cheese* | *E. coli Pasteurised Cheese* | *L. monocytogenes Raw Cheese* | *L. monocytogenes Pasteurised Cheese* |
|---|---|---|---|---|
| 4 | 0.0048 | 0.003 | 0.0009 | 0.0013 |
| $4_{biphasic}$ | - | - | *0.0015* | - |
| 7 | 0.0051 | 0.0031 | 0.0008 | 0.0012 |
| $7_{biphasic}$ | - | - | *0.0014* | - |
| 10 | 0.0053 | 0.0034 | 0.0011 | 0.001 |
| $10_{biphasic}$ | - | - | *0.0018* | - |
| 15 | 0.0065 | 0.0054 | 0.0026 | 0.0019 |
| **25** | 0.0108 | 0.0079 | 0.0068 | 0.0044 |

The inactivation rates in Table 3.1 were presented as Arrhenius plots to assess the strength of the relationship between inactivation rate and temperature. The Arrhenius plots for both species and for cheeses made from either raw or pasteurised milk, and considering the alternative rates based on the steepest part of the inactivation curve for *L. monocytogenes* in raw milk, is shown in Figure 3.4.

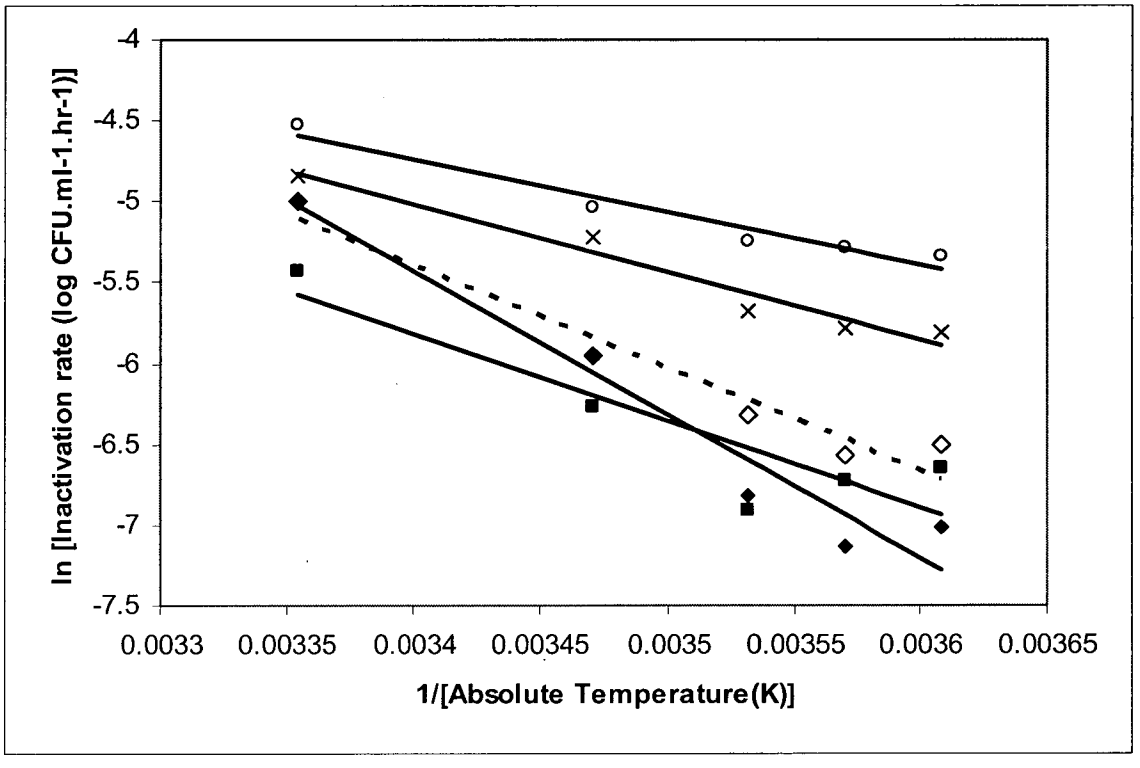


**Figure 3.4.** Arrhenius plot showing the effect of temperature (4-25°C) on the rate of inactivation of pathogens in a semi-hard cheese made from either raw or pasteurised milk. *L. monocytogenes* raw milk (♦), *L. monocytogenes* pasteurised milk (■), *E. coli* raw milk (○) and *E. coli* pasteurised milk (x). The dashed line represents alternative *L. monocytogenes* rates based on the steepest part of the inactivation curves in raw milk (◊) (*see text* for explanation). The fitted parameters of the regression equations fitted to these data are listed in Table 3.1.

The slopes, *y*-intercepts, $R^2$ and Root Mean Square Error (RMSE) for the Arrhenius model fitted to each species and cheese type (i.e. raw milk, or pasteurised milk) are detailed in Table 3.2 and indicate that the data for each species are well explained by the Arrhenius equation with an average $R^2$ for *L. monocytogenes* and *E. coli* of 96% and 89% respectively. The steepness of the slope of the fitted line in the Arrhenius plot indicates the strength of the influence of temperature.

**Table 3.2.** Slope, *y*-intercept and correlation co-efficient of Arrhenius models for the rate of inactivation of *L. monocytogenes* and *E. coli* in semi-hard cheese made from raw or pasteurised milk.

| | *E. coli* Raw Cheese | *E. coli* Pasteurised Cheese | *L. monocytogenes* Raw Cheese | *L. monocytogenes* Raw Cheese (biphasic) | *L. monocytogenes* Pasteurised Cheese |
|---|---|---|---|---|---|
| slope | -3256.5 | -4154.6 | -8858.2 | -6420.9 | -5357.7 |
| *y*-intercept | 6.335 | 9.103 | 24.684 | 16.426 | 12.394 |
| $R^2$ | 0.96 | 0.96 | 0.95 | 0.95 | 0.82 |
| RMSE | 0.182 | 0.226 | 0.063 | 0.146 | 0.078 |

Figure 3.4 shows both Arrhenius plots for *L. monocytogenes* in raw milk semi-hard cheese. The plot with the dashed line and open symbols was produced from inactivation rates taken from the steepest part of the inactivation curve for *L. monocytogenes* in raw milk semi-hard cheese at 4°C, 7°C, and 10°C in Figure 3.3 for the reasons explained above. The alternative inactivation rates produce an Arrhenius plot more consistent with the Arrhenius models produced for *L. monocytogenes* in pasteurised semi-hard cheese and *E. coli* in both types of cheese. It is notable that the slopes are smaller, in general, than those determined for *L. monocytogenes* inactivation rates from the broth study (*see* Table 2.2), suggesting a different temperature response. Statistical evaluation of the various datasets was undertaken using the methods described in Section 2.2.3.1.

When the Arrhenius model for *L. monocytogenes* in raw milk cheese based on the slower inactivation rates was used, no significant difference ($p > 0.1$) between the inactivation rates for *L. monocytogenes* in raw or pasteurised cheese was found. Comparison of the Arrhenius models for the inactivation of *E. coli* in raw and pasteurised cheeses reveals there is a significant difference ($p < 0.05$) in the absolute rate of inactivation (i.e. between the intercepts of the equations) however the intercepts on the Arrhenius scale are 7.91 and 7.53, which represents two-fold difference in absolute inactivation rates, but a difference that might be difficult to resolve in practice using existing enumeration techniques..

Examination of Figure 3.4 clearly shows that the absolute rates of inactivation of *L. monocytogenes* and *E. coli* are systematically different at all temperatures. This was tested statistically by pooling the *L. monocytogenes* inactivation data for both

raw and pasteurised cheese (which were shown not to differ significantly) and comparing it against the pooled raw and pasteurised cheese inactivation data for *E. coli* (also statistically shown to not differ significantly) The responses of the both species to the effect of non-thermal temperature is significantly different for both the slope ($p < 0.05$) and the intercept ($p < 0.05$) indicating that the two species respond differently to temperature in inimical environments under non-lethal temperature conditions. Specifically there is a significant difference in the absolute inactivation rates of the two species with *L. monocytogenes* dying significantly slower than *E. coli* in semi-hard cheese. There was no significant improvement in the goodness of fit when a quadratic model was fitted to the pooled *E. coli* data, however, there was a significant ($p < 0.05$) improvement in the fit when a quadratic model was fitted to the pooled *L. monocytogenes* data, as was observed in Chapter 2 for inactivation data for *L. monocytogenes* in inimical broth.

### 3.3.3 Model comparison

Figure 3.5 compares the Arrhenius model described in Section 3.3.1 with the models of Zhang et al (2010), using *L. monocytogenes* and *E. coli* inactivation data in broth at pH 3.5 and $a_w$ 0.90, and the model of McQuestin et al (2009), using *E. coli* data from salami. Figure 3.5 also includes the inactivation data for *L. monocytogenes* in broth at pH 3.5 and $a_w$ 0.90 presented in Chapter 2. The alternative Arrhenius model for *L. monocytogenes* in raw milk semi-hard cheese, shown in Figure 3.4 (based on slower inactivation rates, shown as dotted line) was used for the model comparisons. The models from other published studies are included to enable comparison of the relative and absolute effects of temperature on inactivation rates of vegetative bacteria under conditions where temperature, *per se*, is not a lethal factor. To increase the size of the data sets, the inactivation rates of *E. coli* at each experimental temperature in raw and pasteurised milk cheese, which were shown not to differ significantly, were combined to represent a single data set and an Arrhenius equation was fitted to the combined data set. The same was done for inactivation rates of *L. monocytogenes* in cheese, for the same reasons.

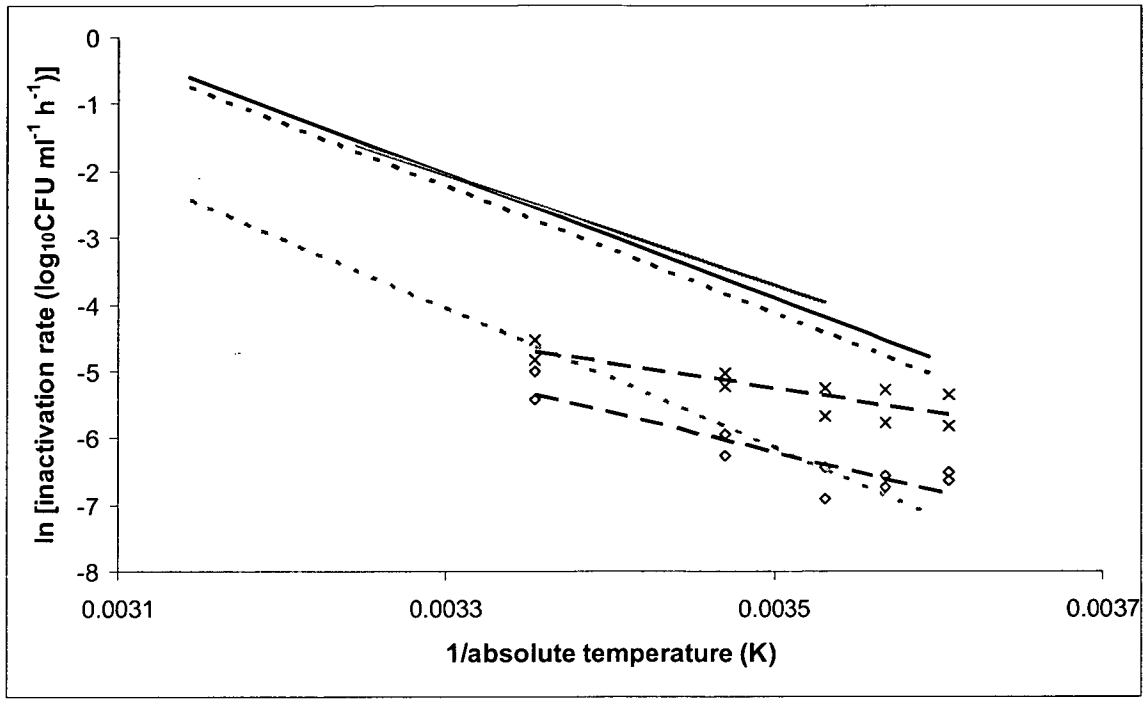


**Figure 3.5.** Comparison of the prediction of Arrhenius models for the non-thermal inactivation of vegetative bacteria in fermented foods and broths at 4 to 45°C. Data was derived from the inactivation of *L. monocytogenes* in semi-hard cheese (large dashed black line and ◊) and *E. coli* in semi-hard cheese (large dashed black line and x). The comparison model data were derived from *L. monocytogenes* (solid grey line) in broth at pH 3.5 and $a_w$ 0.90 (Chapter 2), *L. monocytogenes* (solid, black line) and *E. coli* (dashed, black line) in broth at pH 3.5 and aw 0.90 (Zhang et al. 2010), *E. coli* inactivation in a salami product (dashed, grey line) (McQuestin et al. 2009).

From visual assessment of the data and fitted models in Figure 3.5 the relative effects of temperature on the inactivation of *L. monocytogenes* and *E. coli* in semi-hard cheese are not consistent with the temperature effects seen in other studies (i.e. the slopes of the Arrhenius plots for each organism in cheese are quite different to those seen in the other studies). Examination of the Arrhenius plot for both species in semi-hard cheese indicates that the effect of non-lethal temperature on the inactivation of *E. coli* and *L. monocytogenes* in semi-hard cheese is not as strong as it is for the same species in the other systems as summarised by the various models presented. This can be seen by the shallower slope of the line of best fit in Figure 3.5 to the novel data for inactivation in cheese.

The absolute rates for each organism in semi-hard cheese differ by a factor of 100 to 200% (approximately 0.5 to1 *ln* unit). The effect of temperature on the non-thermal inactivation of *E. coli* is not consistent with that of *L. monocytogenes* or the data

summarised in the other models shown. The absolute rates for the different models differ by up to five factors or two *ln* units. The pooled data set for the inactivation of *L. monocytogenes* in semi-hard cheese was compared to that for its inactivation in inimical broth (see Chapter 2). The inactivation response of *L. monocytogenes* in semi-hard cheese was significantly different ($p < 0.001$) to the inactivation response in broth, both for the slope of the line and the intercept indicating that *L. monocytogenes* is responding differently to temperature in cheese compared to broth. The inactivation rates of *E. coli* in semi-hard cheese were compared to the inactivation rates for *E. coli* in inimical broth derived by Zhang et al (2010). The response of *E. coli* is significantly different ($p < 0.001$) to the inactivation response in broth both for the slope of the line and the intercept indicating that the response to temperature of *E. coli* is systematically differently in Manchego-style semi-hard cheese compared to the response in inimical broth.

### 3.3.4 Inactivation of Exponential Phase *L. monocytogenes* and *E. coli* populations in Raw and Pasteurised Semi-Hard Cheese 15ºC

Inactivation curves for *E. coli* and *L. monocytogenes* at 15°C in both pasteurised and raw milk semi-hard cheeses are shown in Figure 3.6. The cultures used in this experiment were in exponential phase growth rather than stationary phase like the cultures used in the first batch of cheeses. The systematic difference in rate of inactivation between the two species is clear with *L. monocytogenes* surviving for longer periods in both types of cheese than *E. coli* at 15°C. The variability between individual datasets of the same species in different initial growth phases is minimal in comparison to the between species differences. The inactivation rates for each species, cheese type and growth phase, obtained by fitting a linear model to the curves in Figure 3.6, are shown in Table 3.3. Only data relevant to times after curd formation (approximately one hour after inoculation and renneting) were used since it was again apparent that an initial increase in cell density occurred over the first few hours of cheese processing, corresponding with curd formation and removal of the whey.

Inactivation of both exponential phase *L. monocytogenes* and exponential phase *E. coli* at 15°C in semi-hard cheese appears to be faster than the inactivation of those pathogens at the same temperature when they are in stationary phase as shown by

comparing the inactivation curves in Figures 3.3 with those shown in Figure 3.6 and the rates of inactivation in Tables 3.1 and 3.3. For both species, the time taken to inactivate exponential cells is approximately half that taken to inactivate stationary cells.

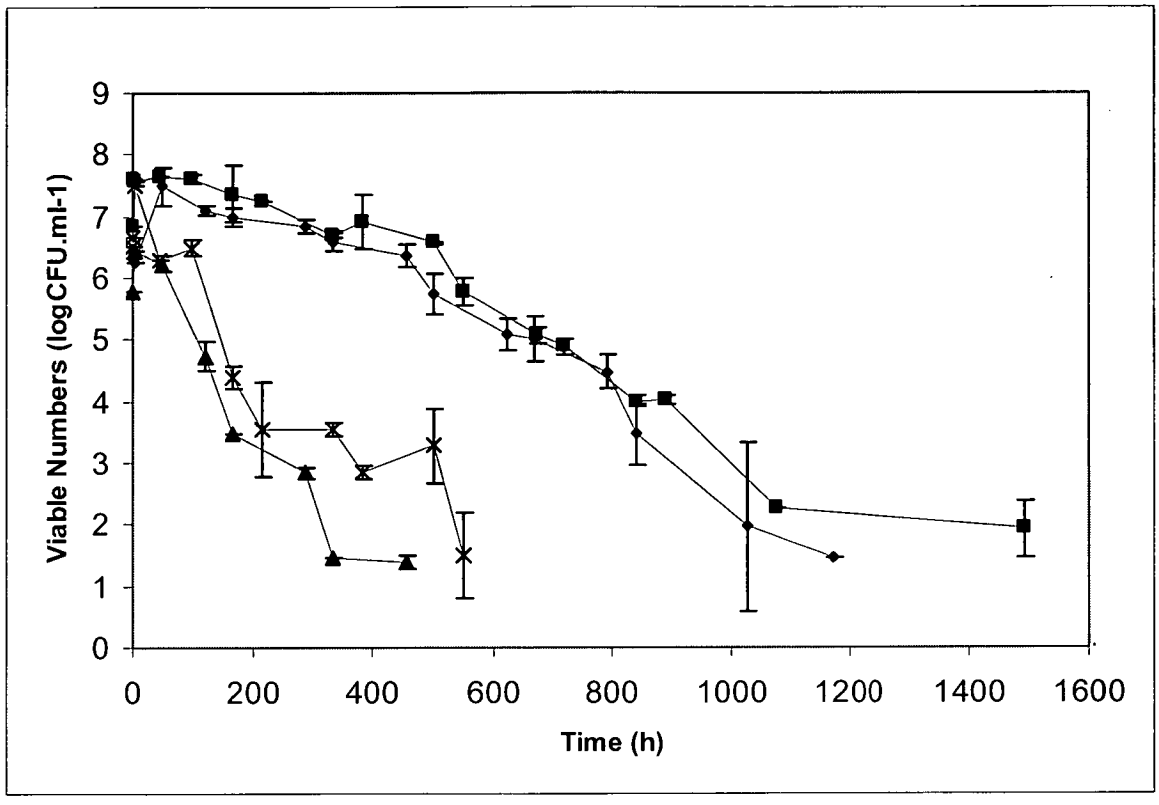


**Figure 3.6.** Non-thermal inactivation curves for *E. coli* M23 in raw cheese (▲) and pasteurised cheese (×) and for *L. monocytogenes* Scott A in raw cheese (♦) and pasteurised cheese (■) stored at 15°C.

**Table 3.3.** Rates of inactivation and $R^2$ values of exponential phase cultures of *L. monocytogenes* and *E. coli* in raw and pasteurised semi-hard cheese at 15°C.

| | *E. coli* Raw Cheese | *E. coli* Pasteurised Cheese | *L. monocytogenes* Raw Cheese | *L. monocytogenes* Pasteurised Cheese |
|---|---|---|---|---|
| Inactivation rate | 0.0122 | 0.0090 | 0.0049 | 0.0042 |
| $R^2$ | 0.93 | 0.87 | 0.89 | 0.93 |

### 3.4 Discussion

The mechanisms of survival or non-thermal inactivation of microorganisms in foods are poorly understood but are assumed to be governed or, at least, potentiated by factors inherent in the food itself or the conditions in which it is stored. The pH and $a_w$ of a food, and the time and temperature of processing and storage, can all be manipulated to minimise the survival of any pathogenic microorganisms. Cheese is a fermented milk product in which the milk sugar lactose is fermented by lactic acid bacteria naturally present in raw milk and those added as a controlled started culture. The $a_w$ of cheese is changed firstly by the coagulation of casein proteins and the consequent expulsion of water and whey proteins and then by salting and drying. The cheese made in the studies described in this chapter is classed as a semi-hard cheese and characterised by a final pH between 4.5 - 4.7 and $a_w$ between 0.91 and 0.92, conditions sufficient in combination to prevent growth of both *L. monocytogenes* and *E. coli.*

Because inactivation is a stochastic process, even though conditions in the cheese are ultimately inimical to bacterial growth, some of the pathogenic bacteria that may be present in raw milk destined for cheese making may survive the fermentation during the early stage of cheese making. Equally, pathogens in some instances may also survive any heat treatment applied to milk prior to cheese making. The majority of microorganisms in, or added to, the milk are trapped in the curd during the process of syneresis while only a small proportion of cells are removed by the expulsion of whey. This has been demonstrated by a number of authors (Goepfert et al. 1968; Dominguez et al. 1987; Yousef et al. 1988; Papageorgiou and Marth 1989a; Yousef and Marth 1990; Buazzi et al. 1992a) and also in the current studies (Figure 3.1) where an increase in bacterial density in the curd was observed during the first two days of manufacture.

The cheese curd is made up of protein micelles, fat globules and a small proportion of free water. The entrapment of bacterial cells in the cheese curd leads to sometimes-erratic data as seen, for example, in Figure 3.3. It was noticed that in some of the sampling instances some cheese pieces yielded no pathogenic cells at all even early in the challenge trial when introduced organisms were still at high levels.

However, at no time throughout the experiments was obvious growth of either challenge organism seen.

The patterns of microbial inactivation of *L. monocytogenes* and *E. coli* in semi-hard cheese shown in Figure 3.3 are relatively consistent with other studies undertaken in analogous aqueous systems and foods (McQuestin et al. 2009; Zhang et al. 2010). The inactivation kinetics of *L. monocytogenes* in raw milk cheese, especially at lower temperatures, appears to be different to the same pathogen in pasteurised milk cheese or *E. coli* in both types of cheese (see Figures 3.3). *L. monocytogenes* stays at high levels in the cheese for over three months at 4°C before numbers begin to decline giving the inactivation curve a biphasic appearance. This is also the case with the inactivation at 7°C and 10°C with numbers staying elevated for just over two months at both temperatures. This effect was not seen in the inactivation of *L. monocytogenes* in pasteurised milk semi-hard cheese. *L. monocytogenes* may have gained some protective advantage from native organisms or other factors active in the raw milk at lower temperatures but it is not clear what that advantage, or the mechanism(s) involved, may be from this study. It does not appear that the pH or $a_w$ of the raw milk cheese changed in any way coincident the time inactivation started (see Figure 3.2). Further investigation is required to determine if the biphasic inactivation seen in this series of experiments is repeatable and to determine what the causes may be. The same effect is not seen in the inactivation kinetics of exponential phase *L. monocytogenes* in raw or pasteurised cheese (Figure 3.6).

In fermented foods such as salamis and cheeses, as the fermentation proceeds the decreasing pH, increasing organic acid levels and reduced $a_w$, can eventually prevent the growth and potentiate the subsequent inactivation of bacteria in these foods. If growth is inhibited the levels of the factors preventing growth are relatively unimportant to the rate of microbial inactivation. Temperature becomes the main factor governing microbial inactivation in these instances and the process has been termed non-thermal inactivation (Ross et al. 2008). This is evidenced by the inactivation kinetics seen for both *L. monocytogenes* and *E. coli* in the semi-hard cheese manufactured in this study. The pathogens were not able to grow in the cheese and were inactivated with the fastest inactivation rates witnessed at the highest temperature tested. It is notable that the slopes of the lines fitted to the

Arrhenius models in Figure 3.4 and show in Table 3.2, are a lot smaller, in general, than those from the broth studies in Table 2.1 (Chapter 2), suggesting a different temperature response. The relative and absolute responses in cheese for both pathogens are also significantly different to their responses in broth and could be interpreted as death occurring by some mechanism other than what occurs in the broth. Further studies are required to assess this. The inactivation studies in fermented meats for *E. coli* by McQuestin (2006) and McQuestin et al (2009), however, did not appear vastly different. The response for *E. coli* inactivation in fermented meats compared with broths was slower (the absolute rate was different) but the relative effects of non-thermal temperature were similar.

The semi-hard cheese produced for this study had pH levels (4.5-4.7) which, on their own, are not sufficient to preclude the growth of both *E. coli* and *L. monocytogenes* (Tienungoon et al. 2000; Ross et al. 2004). In combination with lactic acid or reduced $a_w$ the environment in the cheese become growth-prohibitive for both pathogens (Salter et al. 2000). The $a_w$ of the cheese was stabilised at 0.91 - 0.92 due to vacuum packaging. Had the cheeses been air-ripened and then covered in wax as they would in a commercial manufacturing process the $a_w$ would, potentially, have dropped to lower levels. Despite this the $a_w$ reached in this study was sufficient to prevent the growth of *E. coli* and likely to prevent the growth of *L. monocytogenes* especially in conjunction with the inhibitory effect of low pH and the presence of organic acids (Ross et al. 2000; Salter et al. 2000). It was expected, therefore, that the temperature of ripening would have the greatest effect on the inactivation of *L. monocytogenes* and *E. coli* in semi-hard cheese made from both raw and pasteurised milk.

To test this hypothesis, the inactivation rates of *L. monocytogenes* and *E. coli* in semi-hard cheese were plotted on Arrhenius coordinates (Figure 3.4). Taking into account the biphasic inactivation kinetics of *L. monocytogenes* in raw milk cheese an alternative Arrhenius model was also produced using inactivation rates determined at the sample point where inactivation was observed to have begun and coinciding with the steepest part of the inactivation curves for 4°C, 7°C and 10°C for the (Figure 3.3b). The Arrhenius models generated for the inactivation of *L. monocytogenes* and *E. coli* in semi-hard cheese appear to be consistent with the observation in the

literature. A significant difference was found between the rates of inactivation for *E. coli* in raw milk and pasteurised milk but no significant difference was found for the same comparison for *L. moncytogenes*. This was unexpected and it is not known whether the natural constituents in raw milk played any part since *E. coli* inactivation in raw milk was faster than in pasteurised milk. The effects of temperature are different for the two species when the pooled datasets for inactivation were compared. Zhang et al (2010) found no significant difference between the response of *L. monocytogenes* and *E. coli* to inactivation under non-thermal temperature in inimical broth. The current work in cheese does not support the findings of Zhang et al (2010), however, further investigations of the kinetics of non-thermal inactivation for these two species would be required to further evaluate this conclusion. Importantly, no data is available at 30 to 40 °C for the cheese models as in salami models used for comparison, since maturation of cheese at such temperatures produced adverse spoilage effects that affected the enumeration of pathogenic bacteria. In effect the plot in Figure 3.5, is only the part of the Arrhenius plot that shows the tailing curvature that was identified in Chapter 2 but this is obscured by presenting the modelled data for the models by McQuestin (2006) and McQuestin et al (2009). Currently access to the raw data from those studies is not available but analysis of the raw data for those models may further elucidate whether this is the case.

Of note is the analysis that suggests that a more complex model may better explain the inactivation response to temperature of *L. monocytogenes* in cheese. As with the broth data in Chapter 2, and the data used for meta-analysis, the *L. monocytogenes* inactivation rates in Figure 3.4 show upwards tailing curvature at low temperature, a recurrent theme in the data presented in this thesis. A quadratic model fits the *L. monocytogenes* data significantly better than a linear model. This is not the case, however, for *E. coli*. That this curvature has now been seen in the broth dataset, the literature survey data set and now the cheese challenge trial results points to it not being an experimental artefact or a bias in the low temperature data, but rather a real physiological response in *L. monocytogenes* that is not evident in the responses of *E. coli*. The basis of that response remains to be ascertained.

The work presented in this chapter was undertaken in a model cheese whose manufacture did not follow commercial ripening practices since unripened cheese pieces were vacuum packed shortly after moulding. This work was undertaken as a first step in the analysis of the non-thermal inactivation of pathogens in a fermented food other than salami. Inoculation of *L. monocytogenes* and *E. coli* into separate batches of cheese was used to identify differences between strains independent of any possible species interaction. The results obtained in this work led to the design of the work included in Chapter 4, namely collaboration with a commercial cheese-maker and manufacture of cheese according to the cheese-makers' commercial manufacturing protocol.

## 3.5 Conclusion

A number of recent studies of the inactivation of *E. coli* in fermented meats have found that within the range of temperature, pH, and $a_w$ found in fermented meat processes, rates of inactivation of *E. coli* in these products were dominated by the affect of temperature (Ross and Shadbolt 2001; McQuestin et al. 2006; McQuestin et al. 2009). Zhang et al. (2010)showed that once environmental conditions become growth preventing for vegetative bacteria, temperature within the biological range exerts the greatest influence upon the loss of bacterial viability regardless of species. The work presented in this chapter was designed to investigate the inactivation of *L. monocytogenes* and *E. coli* in response to non-thermal temperature in a fermented semi-hard cheese. Significant differences between the two species in cheese and between both species and equivalent inactivation in broth were found and suggest that the response of vegetative bacteria to inactivation in inimical environments may not be consistent, i.e. firstly that the effect of non-thermal inactivation may not independent of species and, secondly, that inactivation in broths may be systematically different to inactivation in foods.

## Chapter 4. Inactivation of Pathogenic Bacteria during Production of Raw Ewe's Milk, Roquefort-style, Cheese

### 4.1 Introduction

Blue cheeses are a class of semi-hard cheese characterised by the growth of the mould *Penicillium roqueforti* in fissures and piercing channels throughout the cheese. Mould growth gives blue cheeses a characteristic appearance and intense flavour (Cantor et al. 2004). Roquefort cheese is a traditional French blue-veined cheese made from raw ewe's milk, ripened with the mould *P. roqueforti*, and subjected to a ripening period of 15-25 days in the natural aired caves of the Roquefort-sur-Soulzon region (Cantor et al. 2004). Further ripening is then carried out in controlled temperature rooms commonly at temperatures of 2-6°C (Anonymous 2005). The sale of Roquefort in Australia was recently approved.

An application, on behalf of French manufacturers and exporters of Roquefort cheese, was received in 2004 from the French Government (Ministry of Agriculture, Food, Fisheries and Rural Affairs) to amend Standard 2.5.4 - Cheese, of the Australian New Zealand Food Standards Code to permit the sale of Roquefort cheese in Australia (Anonymous 2005). While the Code requires that only pasteurised milk be used for cheese production to manage microbiological hazards, it does have provisions to allow the importation and sale of raw milk cheeses that have been assessed to have equivalent levels of safety as cheeses made from pasteurised milk (Anonymous 2005). A safety assessment to determine if Roquefort cheese can be produced to an equivalent level of safety as cheese made from pasteurised milk was undertaken and Roquefort was approved for import and sale in Australia in 2005 (Anonymous 2005). The microbiological limits set out in Standard 1.6.1 of the Australian Food Standards Code (FSANZ 2009) require that Roquefort cheese must have no detectable levels of *L. monocytogenes* or Salmonella. Additionally, the level of *E. coli* should not exceed 10 per gram, though a maximum level of 100 per gram may be allowed for 1 in 5 samples (Anonymous 2005).

A review of food-borne illness outbreaks associated with raw milk cheeses found that Roquefort cheese has not been implicated in any outbreaks of food-borne illness

(Anonymous 2005). As discussed in previous chapters, several factors are involved in controlling the growth of bacteria in cheese including pH, temperature, salt, and $a_w$ of the cheese. While each has an impact, it is their combined hurdle effect, which influences the growth and survival of pathogens in cheese. Roquefort cheese has an average $a_w$ of 0.92, contains 3% salt and, after prolonged ageing (90+ days), achieves a final pH in the range 6.0-6.5 (Anonymous 2005). In France the cheese making process is heavily monitored to minimise and/or detect potential contamination with pathogenic bacteria, particularly *L. moncytogenes*. These controls, together with the effects of processing on potential pathogenic bacterial contaminants and the fact that Roquefort has not been implicated in any food-borne illness outbreaks, are considered by Australian food safety authorities to provide acceptable levels of protection of public health.

Papageorgiou and Marth (1989a) made experimental blue cheese with *P. roqueforti*. The average salt and water values were 4.5% and 38.9%. No growth of *L. monocytogenes* occurred at the pH values reached at the end of aging (pH 5.5-6.5). A number of batches of blue cheese were manufactured for that study and several reached pH levels marginally above pH 5, indicating that proteolysis and/or growth of moulds may not have proceeded as would be usual for blue mould cheeses. The pH of mould-ripened cheeses increases (to between pH 6-7) later in maturation (Ryser 1999; Chamba and Irlinger 2004) because the ripening moulds *P. camemberti* and *P. roqueforti* are able to utilise lactic acid as a carbon source and because their growth leads to proteolysis of cheese (which releases ammonium ions and increases the pH). It has been argued that the increase in pH may allow *L. monocytogenes* to grow, particularly if the water activity of the cheese is at a level that is 'marginal' for growth prevention.

Challenge studies undertaken by the Institut Pasteur de Lille and the Ecole National Veterinaire Toulouse (Anonymous 2005) concluded that the processes of manufacture of Roquefort cheese makes it unlikely pathogens will survive or proliferate. Food Science Australia undertook a qualitative risk assessment to categorise the risk from potential pathogens in Roquefort cheese (Anonymous 2005) and concluded that during manufacture of Roquefort cheese, pathogens, if present, would be unlikely to survive or grow.

The results from the initial cheese trials in Chapter 3 established that non-thermal inactivation of pathogens is possible in fermented cheese and led to the development of further trials in commercially produced cheese. The study described in this Chapter was undertaken on behalf of Grandvewe Cheeses, Woodbridge, Tasmania, to determine the robustness of a raw sheep's milk Roquefort-style cheese to accidental contamination with bacterial pathogens. Raw ewe's milk was deliberately inoculated with high levels of three strains of each of *L. monocytogenes* and *E. coli*. One strain each of *L. monocytogenes* and *E. coli* (Scott A and M23 respectively) we common to the cheese trials undertaken in Chapter 3. Additional strains were included to provide a more accurate representation of multiple species profiles that may be present in a real-world contamination event. Six batches of cheeses were prepared in triplicate under laboratory conditions and stored at five temperatures in the range 4°C to 25°C. Cheese manufacture was supervised by Grandvewe's chief cheese-maker, Ms. Diane Rae, using methods typical of a small-scale commercial processor. In addition, and upon request of the cheese-maker, raw milk used in the cheese was sent to a commercial testing laboratory for assessment of contamination with pathogenic bacteria. Fat and protein levels in cheese were also assessed, by an independent NATA accredited laboratory, for possible correlation with changes in numbers of introduced challenge bacteria in the cheese. Changes in levels of the introduced challenge organism were modelled using simple linear regression, where appropriate, and rates of changes for populations of both species plotted as a function of temperature using Arrhenius co-ordinates. Water activity and pH were also assessed throughout the fermentation and maturation periods to ascertain the potential influence of these factors on the fate of the introduced pathogens during the cheese-making and ripening processes. All trials were followed for the equivalent of the normal maturation time for the cheese so that the effect of pH changes in the cheese (specifically pH returning to neutrality later in ripening) could be assessed.

## 4.2 Materials and Methods

### 4.2.1 Bacterial Strains and Media

The inactivation kinetics of three *L. monocytogenes* and three *E. coli* strains in Roquefort-style cheese made from raw ewe's milk were assessed. The challenge organisms included *L. monocytogenes* strains Scott A (a known pathogenic strain widely used in research), 79-2759 and 79-0430 (both of which were originally isolated from sheep's milk). The *E. coli* strains studied were: M23 (a well characterised, acid tolerant, and *non*-pathogenic strain), MG1655 (a K12 derivative widely used in laboratory research and R31 (a verotoxigenic strain isolated from a clinical case). All strains were obtained from the School of Agricultural Science Culture Collection (University of Tasmania). Strain R31 was originally isolated from a clinical case by staff of the Clinical School of the University of Tasmania and provided by Dr. Silvana Bettiol. All strains were maintained in nutrient broth (Oxoid CM1, Adelaide, SA, Australia) containing 30% glycerol (Sigma-Aldrich, Melbourne, VIC, Australia), at -80°C. Strains were recovered on Brain Heart Infusion agar (BHA; Oxoid CM225 to which 15g $l^{-1}$ agar was added) by incubation at 37°C for 24h. Three freeze-dried mesophilic LAB starter cultures were used for cheese production, The LAB preparations were; MM100, LM057, LH100 and MD88 (Danisco, Denmark). LAB were recovered by placing 200 mL of sterile UHT processed milk into a 250 mL Erlenmeyer flask incubated at 30 (±1.0) °C in a shaking water bath (Ratek Instruments, Boronia, VIC, Australia) for one hour. *P. roqueforti* mould spores in a liquid preparation (obtained from Cheeselinks, Vic, Australia) were also added to the sterile milk with the LAB starters.

### 4.2.2 Inactivation of *L. monocytogenes* and *E. coli* in raw ewe's Roquefort-style cheese at 4, 7, 10, 15, and 25ºC

#### *4.2.2.1 Preparation of Stationary Phase Populations of* L. monocytogenes *and* E. coli

To prepare experimental inocula, *L. monocytogenes* and *E. coli* cells were removed from the surface of a thawed stock culture (stored at -80°C) using a sterilised inoculation loop, plated to BHA supplemented with 0.1% sodium pyruvate (Sigma, USA) (BHAP) and incubated for 24 (±0.25) hours at 37 (±0.5) °C. After incubation, five colonies of each bacterial strain on BHAP were inoculated to 100 mL Tryptone

Soya Broth (Oxoid, CM0131) containing 0.6% yeast extract (Oxoid, CM0019; TSB-Ye) in a 250 mL Erlenmeyer flask and incubated statically at 37 (±0.5) °C for 24 (±0.25) hours to achieve stationary phase cultures with viable counts of approximately 9.0 log CFU.mL$^{-1}$.

One mL aliquots of each stationary phase *E. coli* strain were transferred to one 15 mL sterile tube. Cells were pelleted by centrifugation at 5000 rpm for 10 minutes at room temperature in a Universal 16A centrifuge. The supernatant was decanted and one mL of raw milk used to resuspend the cell pellet. The cell suspension was then added to the bulk milk. The same process was performed for the three *L. monocytogenes* strains. Immediately following inoculation a 1 mL aliquot was withdrawn for enumeration by spread plating onto selective agar (see Section 3.1.1.3).

#### *4.2.2.2 Preparation of Raw and Pasteurised Semi-Hard Cheese Curds Using Stationary Phase Populations*

Unpasteurised ewe's milk was obtained within an hour of milking from Grandvewe Cheeses, Woodbridge, Tasmania, delivered to the laboratory by the cheese-maker, and used for cheese making within 3-4 hours of milking. Upon receipt of the raw milk, 100mL of raw milk was sent to a NATA-accredited (No. 854) testing laboratory (Lactos Pty. Ltd., Burnie, Tasmania). The testing facility complies with the requirements of ISO/IEC 17025:2005 and tested the raw milk samples for: butterfat and protein, total Coliforms, coagulase positive *Staphylococcus aureus*, *Listeria* spp (*L. monocytogenes* confirmation was conducted using VIDAS if required), and *Salmonella*. In all, only three samples (out of five) of milk used for experimental cheese making were analysed for background bacterial contamination and protein/butterfat levels since an account with the testing laboratory had not yet been set up when the initial two batches of cheese were manufactured (see Tables 4.1a and b). Two milk samples were sent for assessment of protein and butterfat levels, by the cheese maker, in the weeks between production batches of laboratory cheese trials and that data was included in this Chapter to show how protein and butterfat levels changed over the week.

Ten litres of raw ewe's milk were used for each cheese batch, and milk for cheese making was not mixed with any other raw milk. Cheeses were made at approximately two to three week intervals. In all, five batches of cheese were made for storage at one of the five experimental ripening temperatures making five batches of cheese made in total over a twelve week period. Milk for cheese making received no heat treatment prior to the commencement of cheese production. The LAB starter cultures and mould spore suspensions were placed in an Erlenmeyer flask with 200 mL of sterile UHT milk and incubated in a shaking water bath at 30 (±1.0) °C for 1 hour prior to being added to the bulk milk. Immediately prior to commencement of cheese-making, a 2 mL aliquot of the raw ewe's milk was withdrawn from the bulk milk for pH measurement using a model 250A pH meter (Orion Research Inc. USA) fitted with an Activon AEP 433 flat tip probe (Activon Scientific Products Co. Pty. Ltd., Australia). The pH meter was calibrated prior to use according to the manufacturer's instructions with standard pH solutions (pH 7.00 and pH 4.00). The bulk milk was added to a 16 L sterile stainless steel saucepan suspended inside a larger 20 L saucepan filled with water and warmed on a hotplate. Once the raw milk achieved a temperature of 34°C (±1.0), starter cultures, mould and challenge organism cultures were added to the milk. Non-animal rennet (Cheeselinks, Australia) was added at the rate of 1.5 mL per 10 L of milk and rennet-to-cut time was 2 hours. The curd was cut into approximately 1.0 cm cubes and allowed to sit for 5 minutes. Curd was then cooked with the temperature rising from 32°C to 38°C (±1.0) over a 60-minute period. The curd was also stirred continuously during the cook period. Curds were poured into one of three sterile cheese hoops in approximately equal amounts making three individually hooped cheeses from each 10L batch of milk. Whey was collected, disinfected by boiling for 30 minutes and addition of chlorine bleach disinfectant, and discarded. Curd masses were turned at half hourly intervals back into the hoop over a 2-hour period. The three hooped cheeses were then left at 20°C overnight before all being placed into one of five experimental ripening temperatures (4, 7, 10, 15, or 20 °C). After three days cheeses were removed from the hoops and dry salted at a rate of 24 g NaCl per kilogram of cheese on the top and sides only. Five days after inoculation the cheeses were salted (with the same rate of NaCl as day 3) on the bottom. Cheeses were pierced on the twelfth day after fermentation, then wrapped in aluminium foil, and placed onto

sterile plastic cheese racks placed inside 40L sterile plastic-lidded storage containers on day twenty-two after the fermentation.

Water activity ($a_w$) of the cheeses was measured periodically using an Aqualab CX2 dew point instrument (Decagon Devices, Pullman Washington, USA). The pH was measured when $a_w$ was measured. Three replicates of cheese were placed at each experimental temperature. Samples for $a_w$ and pH measurements were taken randomly from each of the three cheese replicates at each temperature and the values averaged.

#### *4.2.2.3 Sampling and Enumeration of Challenge Organisms*

Cheese was sampled as described in Section 7.3 of Australian Standard 1766.3.15-1994 (FSANZ 2009), i.e. Method 3.15 Examination of specific products-Cheese. Cheese samples (~10g) were transferred to filter stomacher bags (LabServ) and mixed nine parts (wt/wt) (±18.0) mL of pre-warmed (40-45°C) 20g/L sodium citrate solution and 0.1% peptone solution. Thus 90 (±18.0) mL of solution were added to the sample. Samples were homogenised for 3 minutes at 20°C in a stomacher blender (Colworth Stomacher 400). On each sampling day, duplicate cheese samples per treatment were analysed. Appropriate serial dilutions in 0.1% peptone were prepared.

*L. monocytogenes* was enumerated by surface plating either 50, 100 or 250 µL volumes of appropriate dilutions using an Autoplate 4000 spiral plater (Spiral Biotech Inc., Bethesda, MA, USA) onto PALCAM Agar (Oxoid CM0877 with SR0150 supplement) and Listeria Selective Agar (Oxford Formulation; Oxoid CM0856 with SR0140 supplement) and counting colonies that had morphology consistent with typical *L. monocytogenes* on these media after incubation. Plates were incubated for 48 hrs at 37 (±0.5) °C. *E. coli* was enumerated by plating appropriate dilutions onto Eosin Methylene Blue agar (Modified) Levine Agar (Oxoid CM0069) and counting colonies with morphology consistent with typical *E. coli* on this medium. Plates were incubated for 24 hrs at 37 (±0.5) °C. All selective/differential media were made in accordance with the manufacturer's

instructions. As in Section 3.2.2.5 biochemical confirmation of counted colonies was not undertaken.

Numbers of challenge organisms at each sampling time were plotted as $Log_{10}CFU.g^{-1}$ *vs.* time. From these plots, inactivation rates were determined by linear regression of the data. Inactivation rates were then plotted on Arrhenius plots (*ln*(inactivation rate) *vs.* 1/temperature[K]) to determine the temperature dependence of inactivation rate. Significance of temperature, as a factor influencing inactivation rate, was estimated by calculating $R^2$ values for both *L. monocytogenes* and *E. coli* data sets. The inactivation rates for each organism at each temperatures were calculated from the data relevant to times only after the curd formed.

#### *4.2.2.4 Comparison of Arrhenius models*

The Arrhenius model generated for non-thermal inactivation of *L. monocytogenes* and *E. coli* in this study was compared with: i) the non-thermal Arrhenius models presented in Chapter 2 for *L. moncytogenes*; ii) the model for *L. monocytogenes* and *E. coli* presented in Chapter 3, ii) those generated by Zhang et al (2010) for *L. monocytogenes* and *E. coli* and iv) McQuestin et al (2009) for *E. coli*. To assess whether the rates of inactivation response to temperature were significantly different between species or environments (e.g., broth vs. cheese, semi-hard cheese vs. blue milk cheese) the statistical methods described in Section 2.3.3.1 (Chapter 2) was used.

## 4.3 Results

### 4.3.1 Milk composition and background contamination

Analysis of raw milk composition by the NATA accredited laboratory was undertaken on five occasions and for microbial contaminants on three occasions between December 2007 and February 2008. The results are summarised in Table 4.1 a and b. *Listeria* and *Salmonella* spp. were not detected in any of the three samples submitted for testing, therefore, VIDAS screening was not required. Coagulase positive *S. aureus* and *E. coli* were detected but levels were low in all samples.

**Table 4.1a.** Analysis of milk composition.

| Date | Milk Fat (%w/w) | Protein |
|---|---|---|
| December 11, 2007 | 5.50 | 4.92 |
| January 3, 2008 | 6.11 | 4.92 |
| January 16, 2008 | 6.17 | 5.48 |
| January 24, 2008 | 8.81 | 5.17 |
| February 23, 2008 | 7.42 | 5.81 |

**Table 4.1b.** Background microbial levels.

| Date | Coliforms ($MPN.g^{-1}$) | *E. coli* ($MPN.g^{-1}$) | Coagulase positive *S. aureus* presumptive ($cfu.g^{-1}$) | Coagulase positive *S. aureus* confirmed (per 0.1g) | *Listeriae* | *Salmonellae* |
|---|---|---|---|---|---|---|
| November 28, 2007 | No milk sent for analysis (protein and butterfat analysed on December 11) | | | | | |
| January 9, 2008 | No milk sent for analysis (protein and butterfat analysed on January 3) | | | | | |
| January 16, 2008 | 4 | 4 | 1000 | ND | ND | ND |
| January 24, 2008 | 23 | 23 | >1500 | ND | ND | ND |
| February 23, 2008 | ND | - | >1500 | 3 | ND | ND |

ND - not detected

### 4.3.2 Inactivation of Stationary Phase *L. monocytogenes* and *E. coli* populations in Raw Ewe's Milk Roquefort-style Cheese at 4, 7, 10, 15, and 25ºC

Inactivation curves for *E. coli* and *L. monocytogenes* at 4, 7, 10, 15 and 20°C in raw ewe's milk Roquefort-style cheeses are shown in Figure 4.1a and b respectively. *E. coli* inactivation was more rapid than *L. monocytogenes* inactivation at every temperature. Figures 4.1a and b show the degree of reproducibility and in almost all cases, the responses of either organism were consistent between replicates at each temperature. Occasional erratic responses can be seen, e.g. Figure 4.1b ii) between 1500 and 2500 hours or Figure 4.1b iv) after 800 hours at 20°C. It is also evident from the inactivation figures for both organisms that while replicates at each time were usually similar, duplicate samples from each replicate were at times inconsistent.

Inactivation rates for *L. monocytogenes* and *E. coli* at each test temperatures were calculated by simple linear regression to the curves in Figures 4.1a and b. It is apparent, however, that an initial increase in cell numbers occurred over the first few hours of processing. As discussed in Chapter 3, this corresponds with curd formation and removal of the whey and is likely to result from concentration of the cells into the curd, rather than being due to microbial growth. Thus, only data relevant to times after curd formation (approximately two hours after inoculation and renneting) were used. Apart from this apparent increase, in no case was growth of the either challenge organism observed. Calculated inactivation rates were presented as Arrhenius plots to assess the strength of the relationship between inactivation rate and temperature. The Arrhenius plots and resulting lines of best fit for both species in Roquefort-style cheeses made from raw ewe's milk are shown in Figure 4.2. The rate of inactivation for each challenge species is presented in Table 4.2. Three rates are presented for each temperature as these are the rates for each of the three cheese replicates placed at each temperature. Of note are the rates of inactivation for *E. coli* at 4°C because they appear to be the same as the observed pathogen inactivation rate at 7 °C.

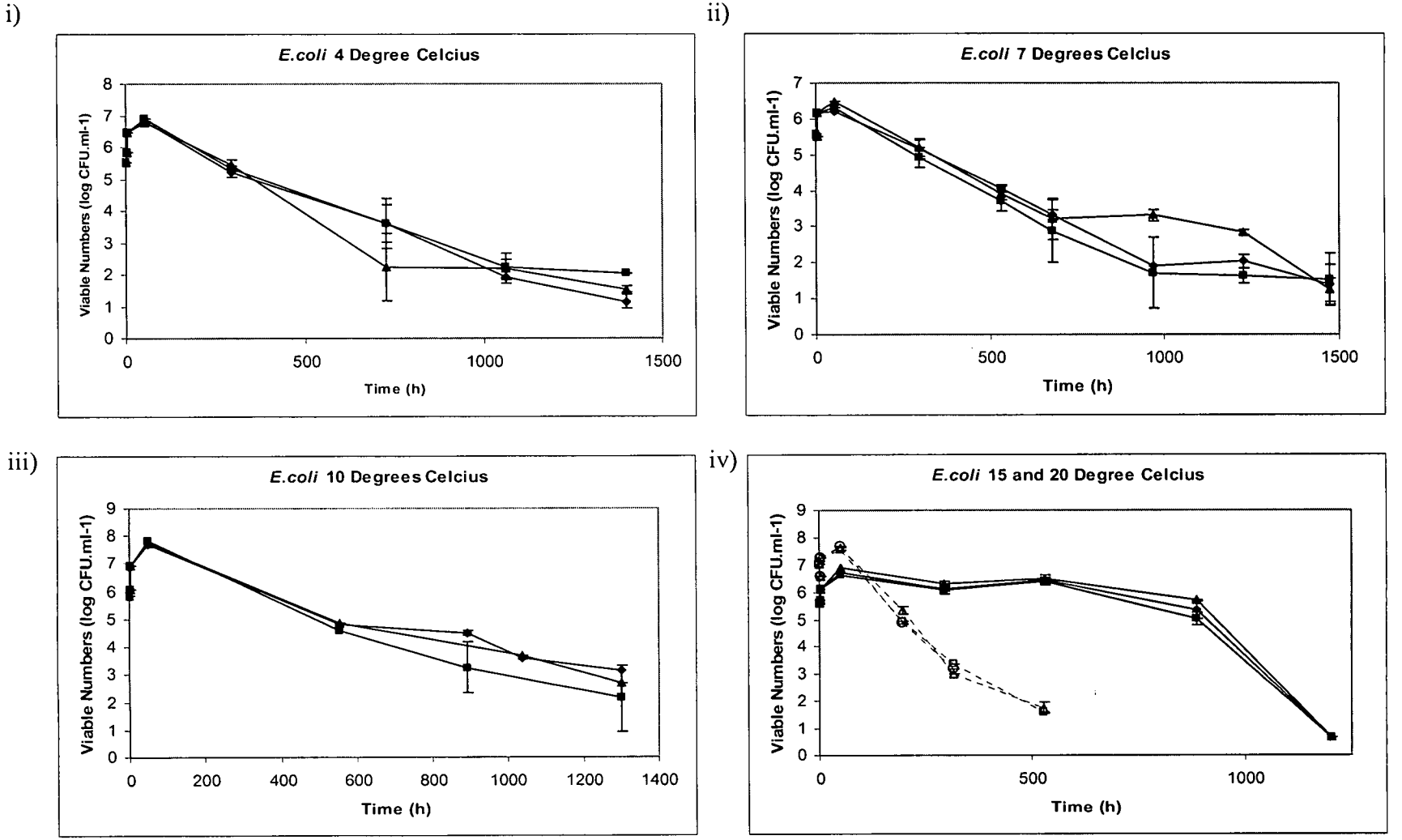


**Figure 4.1a.** Non-thermal inactivation curves for a three strain cocktail of *E. coli* at i) 4°C, ii) 7°C, iii) 10°C, and iv) 15°C (closed symbols) and 20°C (open symbols) in raw ewe's milk Roquefort-style cheese.

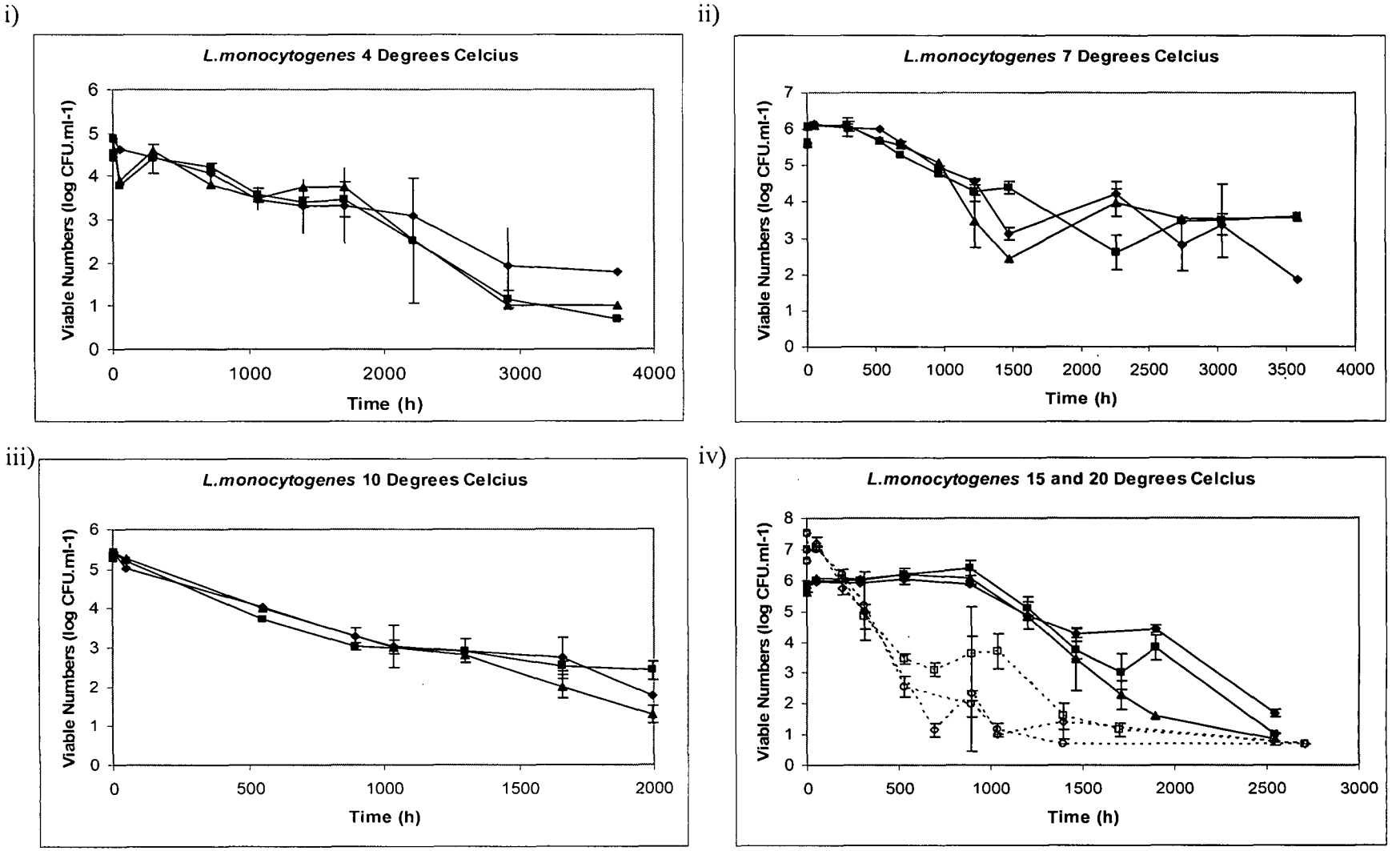


**Figure 4.1b.** Non-thermal inactivation curves for a three strain cocktail of *L. monocytogenes* at i) 4°C, ii) 7°C, iii) 10°C, and iv) 15°C (closed symbols) and 20°C (open symbols) in raw ewe's milk Roquefort-style cheese.

**Table 4.2.** Rate of inactivation ($log_{10}$CFU per hour) of *E. coli* and *L. monocytogenes* in raw ewe's milk Roquefort-style cheese at various temperatures.

| Temperature °C | *E. coli* Inactivation rate | *L. monocytogenes* Inactivation rate |
|---|---|---|
| 4 | 0.004 | 0.0008 |
| 4 | 0.0037 | 0.001 |
| 4 | 0.0042 | 0.001 |
| 7 | 0.0034 | 0.0011 |
| 7 | 0.0035 | 0.0009 |
| 7 | 0.0032 | 0.001 |
| 10 | 0.0027 | 0.0017 |
| 10 | 0.0036 | 0.0016 |
| 10 | 0.003 | 0.002 |
| 15 | 0.0046 | 0.0016 |
| 15 | 0.0046 | 0.0021 |
| 15 | 0.0047 | 0.0025 |
| 20 | 0.0123 | 0.0032 |
| 20 | 0.0169 | 0.0046 |
| 20 | 0.0125 | 0.006 |

The $R^2$ values for the Arrhenius equations fitted to the data indicate that temperature accounts for 87% of the observed *ln*(inactivation rate) for *L. monocytogenes* but only 63% for *E. coli* in Roquefort-style cheeses made from raw ewe's milk. The steepness of the slope of the fitted line in the Arrhenius plot indicates the strength of the influence of temperature and it is clear that there are systematic differences at all temperatures between the challenge species. This relationship was examined statistically and it was found that when the slopes of the two Arrhenius models were compared, both with the *E. coli* 4°C data included and with it removed, the slopes of the two lines were not significantly different ($p > 0.5$) when the 4°C was omitted. When two lines (i.e., a separate slope and intercept determined for each of the two data sets) were fitted (Model 1 in Section 2.2.3.1) and compared to the equation for a single line (both data sets treated as one population) the improvement in the goodness of fit was significant ($p < 0.001$). When the equation for one line was compared to the model for a common slope but using two intercepts (Model 2 in Section 2.2.3.1), Model 2 was significantly ($p < 0.001$) better at describing the data.

Comparison of the fit of Model 3 to Model 2 did not significantly improve the fit indicating that the relative effect of temperature is similar for both species (i.e the slopes of the fitted lines are similar) but that the absolute effect is different (significantly different intercepts). A quadratic line fitted to the data for *L. monocytogenes* significantly improved the goodness of fit ($p < 0.005$) as did a quadratic fitted to the data for *E. coli* (*sans* data at 4°C, $p < 0.001$).

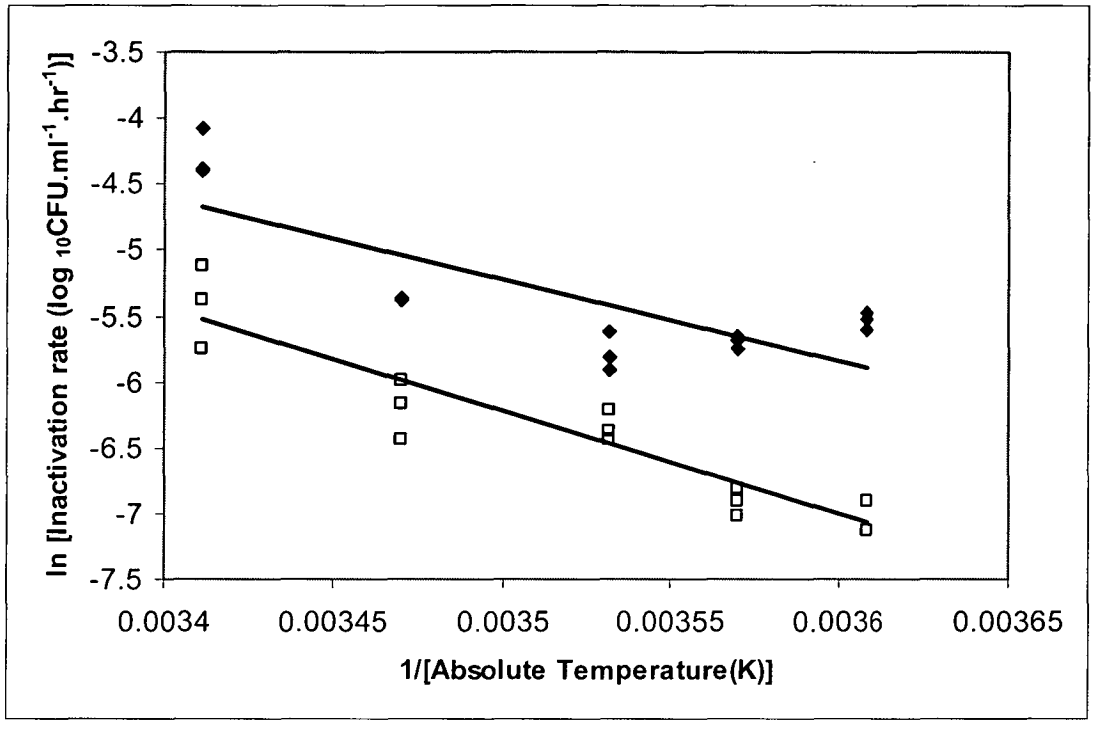


**Figure 4.2.** Arrhenius plot showing the effect of temperature (4-20°C) on the rate of inactivation of *E. coli* (♦) and *L. monocytogenes* (□) in Roquefort-style cheese made from raw ewe's milk. The regression equation parameters are listed in Table 4.2. The regression equations fitted to the data are *E. coli*, $y = -6185.8x + 16.429$ ($R^2 = 0.63$), and *L. monocytogenes*, $y = -7831.7x + 21.184$ ($R^2 = 0.87$)

### 4.3.3 Comparison of Arrhenius Models

Figure 4.3 compares the Arrhenius model generated in Chapter 3 for the inactivation of *L. monocytogenes* and *E. coli* in raw and pasteurised semi-hard cheese with the Arrhenius models developed for the same species in this Chapter. Inactivation rates for *E. coli* and in raw Roquefort-style cheese at 4°C were excluded from the fitting of the regression line in Figure 4.3 because they appear to be anomalously fast. Data were excluded to determine what effect this had on the fit of the model. The model

from Chapter 2 for the inactivation of *L. monocytogenes* in broth with pH 3.5 and $a_w$ 0.90 is also included for comparison. Models from Zhang et al (2010), using *L. monocytogenes* and *E. coli* inactivation data in broth at pH 3.5 and $a_w$ 0.90, the model from McQuestin et al (2009), using *E. coli* data from fermented meats and the model for *L. monocytogenes* in broth at pH 3.5 and $a_w$ 0.90 presented in Chapter 2 are also included. Comparison with other models is useful to examine both the relative and absolute effects of temperature on the inactivation rates of vegetative bacteria under conditions where temperature is not a lethal factor to determine the generality, or otherwise, of the responses described in the published literature.

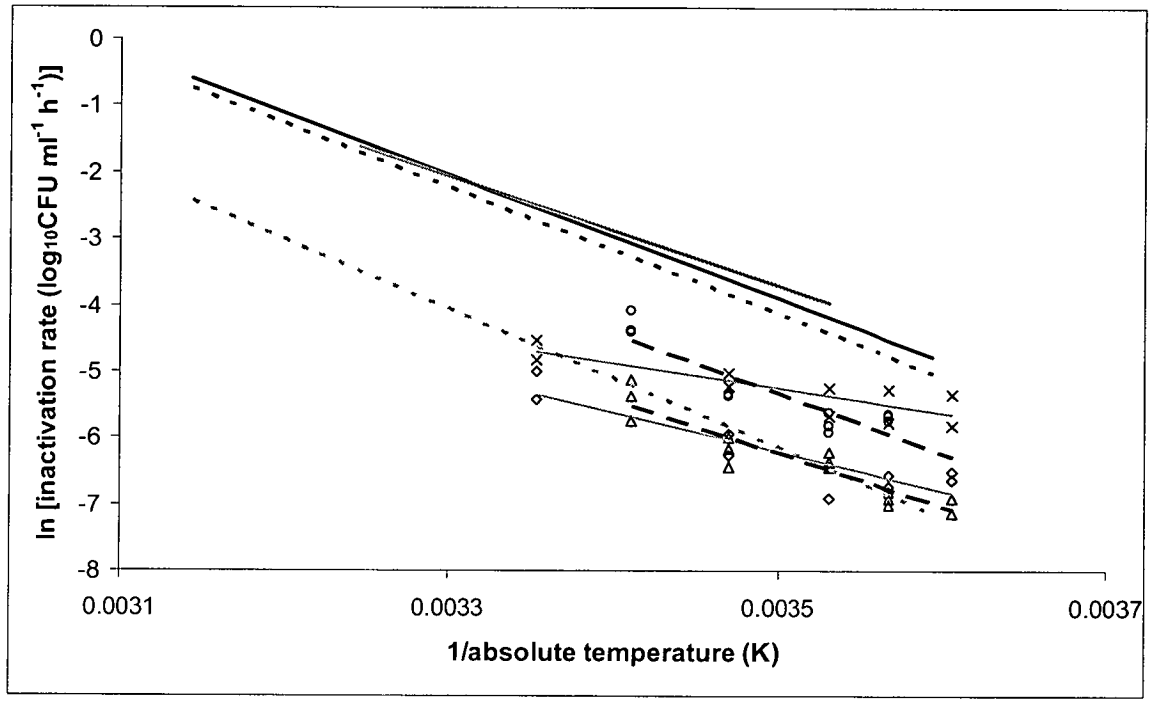


**Figure 4.3.** Comparison of the prediction of Arrhenius models for the non-thermal inactivation of vegetative bacteria in fermented foods and broths at 4 to 45°C. Data from this study are shown: *E. coli* (large dashed black line and o) and *L. monocytogenes* (large dashed black line and Δ) inactivation in Roquefort style raw ewe's milk cheese. (Inactivation rate data for *E. coli* at 4°C were omitted from the regression). Other models are presented for comparison: *L. monocytogenes* (solid, black line) and *E. coli* (dashed, black line) in broth at pH 3.5 and aw 0.90 (Zhang et al. 2010), *E. coli* inactivation in a salami product (dashed, grey line) (McQuestin et al. 2009), *L. monocytogenes* in semi-hard cheese (thin grey line and ◊) and *E. coli* in semi-hard cheese (thin grey line and x) (Chapter 3) and *L. monocytogenes* (solid grey line) in broth at pH 3.5 and aw 0.90 (Chapter 2).

Examination Figure 4.3 indicates that the slopes of the fitted lines of the Arrhenius plot for *L .monocytogenes* and *E .coli* in Roquefort-style cheese are consistent with the slope of the lines of best fit for inactivation data for *E. coli* in fermented meats, broth, and *L. monocytogenes* in broth. The slopes are not, however, consistent with those for the cheese challenge studies described in Chapter 3. The slope of the fitted line for the inactivation of *E. coli* in Roquefort-style cheese was compared to that for the inactivation of *E. coli* in inimical broth from the work of Zhang et al (2010) and to the models fitted to the cheese data from Chapter 3.

Model 1 (two lines) provided a significant improvement ($p < 0.001$) in describing the data when compared to Model 3 (a single line fitted to the data). A significant improvement ($p < 0.001$) was also obtained with Model 2 (one line two intercepts) when compared to Model 3 (a single line fitted to the data). Model 1 did not give a significant improvement compared to Model 2, suggesting that temperature has the same relative effect on the inactivation rate of *E. coli* in the blue cheese as in an inimical broth, but that inactivation in cheese is significantly slower than in broth (different intercepts). The inactivation data for *E. coli* in blue cheese and in semi-hard cheese (*see* Chapter 3) were analysed for the goodness of fit of the three models and it was found that there is a significant difference ($p < 0.05$) in the fit of Model 2 compared to Model 3 indicating that the relative effect of temperature on the inactivation rate in the two cheese types is different.

A similar comparison was made for the inactivation rate of *L. monocytogenes* in Roquefort-style cheese with inactivation rates observed in inimical broths (data presented in Chapter 2) and semi-hard cheese (data presented in Chapter 3). The inactivation rate for *L. monocytogenes* in blue cheese and in semi-hard cheese (from Chapter 3) were fitted to the three models and goodness-of-fit compared. The first analysis considered the full dataset from Chapter 3 with no ommissions. It was found that there is a significant difference ($p < 0.05$) in the fit of Model 1 and 2 compared to Model 3 indicating that the effect of temperature upon the inactivation rate in the two cheese types is different. When the data at 4°C for the semi-hard cheese were ommitted (*see* Chapter 3 for reasons) there was no significant difference between the slopes and intercepts for either data set, indicating that the relative rate of inactivation of *L. monocytogenes* under conditions of non-lethal temperature in

both types of cheese is similar. Chapter 3 resolved that the inactivation rate response to temperature of *L. monocytogenes* in semi-hard cheese was significantly different to that in broth. When the response of *L. monocytogenes* in blue cheese compared to the response in broth from Chapter 2. It was found that there is a significant difference ($p < 0.001$) in the fit of Model 1 and 2 compared to Model 3 indicating that the effect of temperature upon the inactivation rate in the cheese is different to the rate in broth. When the data at 4°C for the broth were ommitted (*see* Chapter 2 for reasons) there was also a significant difference ($p < 0.001$) in the fit of Model 1 and 2 compared to Model 3.

### 4.3.4 pH and Water Activity Changes during Fermentation and Maturation

pH and $a_w$ changes were monitored in cheeses matured at each experimental ripening temperature. Data from all temperatures are shown in Figure 4.4. The pattern of pH change was consistent at all temperatures, despite the temperature difference, but the process is slower at lower temperatures. In all cheeses, the $a_w$ fell after salting and stayed, consistently, at levels in the range 0.86 to 0.92 until the end of sampling.

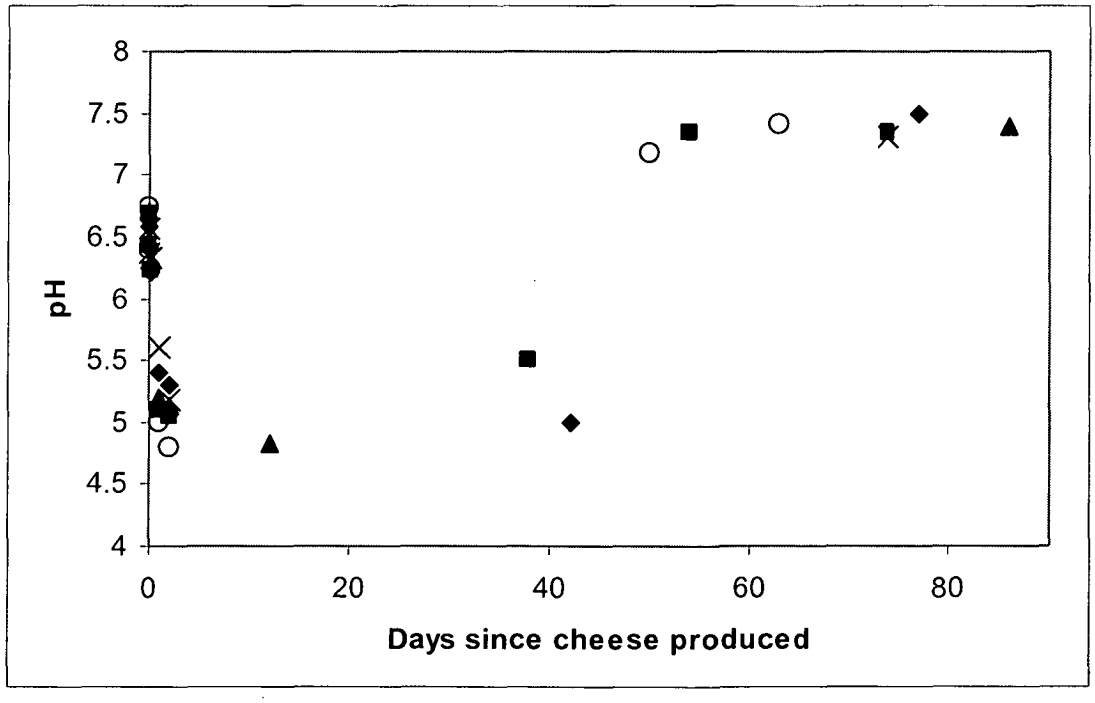


**Figure 4.4** pH changes in Roquefort-style raw ewe's milk cheese matured at 4°C (♦), 7°C (■), 10°C (▲), 15°C (X) and 20°C (○).

## 4.4 Discussion

Some of the inactivation data presented for the challenge pathogens in raw ewe's milk Roquefort-style cheese is erratic, however, no growth of either challenge organism was observed in any of the cheeses ripened at each temperature. The erratic results are most likely due to the complex nature of the cheese matrix and physical changes that occur during manufacture and ripening. Cheese is essentially a mixture of protein, fat and water together with communities of both introduced and indigenous microorganisms. As rennet and acid production cause the proteins in milk to coagulate, some microorganisms become caught within the protein micelles and fat globules, some remain in the water phase while the rest are expelled in the whey, as shown also in this thesis. The random nature of entrapment or clumping of microorganisms in the curd may give rise to the sometimes-erratic raw data. Despite this, the patterns of microbial inactivation in raw Roquefort-style cheese are consistent with those observed in semi-hard cheeses (Chapter 3), that growth of pathogenic bacteria is prevented due to inimical pH and/or $a_w$. It is plausible that non-*Listeria*/non- *E. coli* contaminants may have been present in the raw milk and have been able to grow upon selective plates, however, the high inoculum level used for challenge organisms would negate any effect that contaminants would have upon overall counts. Initial testing of raw milk also showed that prior to the commencement of cheese making contaminants able to grow on the selective agar were not present. An increase in bacterial numbers, between the time of milk inoculation and the time the cheeses were out of the hoops (approximately 50hrs), was observed in all manufactured cheeses as shown in Figures 4.1a and b. This was also observed in the cheese made for the studies reported in Chapter 3 and is attributed to cell entrapment during curd formation (Papageorgiou and Marth 1989a).

Reduction of $a_w$ and pH, through salting and drying and due to the generation of lactic acid by LAB starter cultures, are important aspects of the fermentation process in foods because they retard and may preclude the growth of many pathogenic microbes. The use of effective starter cultures to achieve rapid acid production in cheese is the primary factor responsible for the elimination of pathogens in semi-hard cheeses (Bachmann and Spahr 1995). The raw ewe's milk Roquefort-style cheese employed in the experiments described in this Chapter has a final $a_w$ between 0.86

and 0.92. Water activity in that range is sufficient to prevent the growth of *E. coli* and highly likely to prevent the growth of *L. moncytogenes*. *E. coli* is reported to be unable to grow at $a_w$ below 0.95 (Bell and Kyriakides 1998; Salter et al. 2000) and *L. monocytogenes* is reported to be unable to grow if the $a_w$ is less than 0.92-0.93 (Ross et al. 2000). The $a_w$ of the cheeses in this study is, of itself, enough to prevent the growth of *E. coli* and the combination of bacteriostatic $a_w$ levels together with reduced pH and the presence of lactic acid make it highly unlikely, based on the predictions of growth/no growth interface models such as that by Tienungoon et al (2000), that *L. monocytogenes* could grow, an expectation that is suppoted by the inactivation data presented in Figure 4.1b.

The pH of blue mould cheeses increases later in fermentation (Ryser 1999) and this is clearly shown in Figure 4.4. When *P. roqueforti* grows in cheese it is able to use lactic acid as a carbon source and also causes proteolysis (and subsequent cheese softening), both of which contribute to an increase in pH to levels above 6.5 (Chamba and Irlinger 2004). Papageorgiou and Marth (1989a) found that after 80 days of ripening the pH of blue cheese increased to 5.5-6.0. They also found that *L. monocytogenes* numbers decline throughout ripening until they stabilised once the pH of the cheese reached 5.5. The blue cheese made in that study appears atypical because the pH did not return to a value close to pH 6.5 - 7 as has been well-documented for blue cheese (Coulter et al. 1938; Ryser 1999) and as observed in the work presented in this Chapter (see Figure 4.5). In the cheese made for this study the upper range of $a_w$ is marginal for the prevention of growth of *L. monocytogenes* and the increase in pH may subsequently allow the pathogen to survive and possibly to grow in the cheese. The pH in all experimental cheeses at all temperatures was greater than pH 7 by the end of maturation. Despite this there is no evidence from the survival curves presented (Figures 4.2 a and b), for either *L. monocytogenes* or *E. coli*, that the rate of inactivation is affected by the pH change in the cheeses. This observation supports the hypothesis that temperature is the dominant factor affecting inactivation rate if growth is prevented by other factors. During ripening with *P. roqueforti* proteins, carbohydrates and fats are metabolised and a number of amino acids and free fatty acids are produced. It has been suggested. that metabolites of *P. roqueforti* may have some antimicrobial action (Laporte et al. 1992) but it is not clear from that data whether this occured in the studies described here. As the

mould, *P. roqueforti*, grows the pH of the cheese increases (see Figure 4.4) and it can be concluded that at this point mould metabolites are present in the cheese. pH begins to increase in the blue cheese made in this study between 40 and 50 hours and this corresponds to the maximum population numbers reached in all cheese replicates for both pathogens (raw data not shown) after which point population numbers begin to decline. The increase in population numbers, however, is most likely caused by increasing syneresis of the curd within the first two days of manufacture and the first stage of salting, not from pathogen growth.

As noted in the previous two chapters a number of authors (McQuestin et al. 2006; McQuestin et al. 2009; Zhang et al. 2010) have shown that once pH and $a_w$ reach levels that prevent growth, temperature primarily determines the rate of microbial inactivation for vegetative cells. Figure 4.2 shows the Arrhenius plot for the inactivation of *E. coli* and *L. monocytogenes* in raw ewe's milk Roquefort-style cheese. Temperature accounts for 87% and 63% of the variance in the observed *ln*(inactivation rate) data of *L. monocytogenes* and *E. coli* in Roquefort-style cheeses made from raw ewe's milk. Statistical analysis of the lines of best fit to the ln(inactivation rate) data for *E. coli* and *L. monocytogenes* indicate that the response of *E. coli* in blue cheese is significantly different to that of *L. moncytogenes*. Closer analysis of the inactivation rates for *E. coli* (see Table 4.2) reveals that the inactivation at 4°C is faster than that at 7°C (average inactivation rate for 4°C is 0.004 and for 7°C is 0.0034). It is not known why the inactivation of *E. coli* was faster at 4°C but both pathogens were inoculated together into the cheeses (in Chapter 3 the pathogens were inoculated into different cheeses) which may have affected *E. coli* at lower temperatures or there may have been uncontrolled variation in inoculum preparation. When the 4°C data were omitted from the modelled data the comparison of the fitted lines for the two species were significantly different for the absolute rate of inactivation (different intercepts) but not the relative rate (slope) unlike the results for the two species in Chapter 3. *L. monocytogenes* inactivation was significantly slower that *E. coli* in Roquefort-style ewe's milk cheese. A quadratic equation fitted to the *L. monocytogenes* and *E. coli* data (sans 4°C) provided a significantly better fit to the data than a linear model. As was seen for *L. monocytogenes* in semi-hard cheese (Chapter 3) a more complex model was significantly better able to explain the inactivation response to temperature of both

pathogens in blue cheese. The upward 'tailing' curvature observed in the ln(inactivation rate) data for *E. coli* was not observed by Zhang et al (2010) or McQuestin (2006). Visually, it is not evident in the meta-analysis of McQuestin et al (2009), however, a more detailed examination is required before this conclusion can be drawn with confidence. The upward tailing in the *ln*(inactivation rate) data for *L. monocytogenes* was observed in every dataset in this thesis. It is apparent that at low temperatures (4-5°C) a different response to temperature is observed in the inactivation of *L. monocytogenes* in inimical environments. It would be worthwhile examining if this phenomenon is repeatable in other inimical systems and examining whether some other mechanism (e.g. an acid tolerance response or the transcription of cold-stress genes and proteins) is affecting inactivation responses. Indeed an investigation into the growth behaviour of *L. monocytogenes* at sub-optimal temperature by Bajard et al (1996) found the existence of a "change temperature" between 10 and 15°C, below which the organism grows faster than expected. This unusual behaviour by *L. monocytogenes* may also be evident in its kinetics of inactivation at low temperature.

The same lines of best fit are shown in Figure 4.3 in comparison with the previously mentioned studies as well as the data for *L. monocytogenes* (Chapter 2) and the semi-hard cheese data presented in Chapter 3. *E. coli* inactivation rate in blue cheese (sans data at 4°C) was significantly slower than the inactivation of *E. coli* in laboratory broth in the work of Zhang et al (2010). The relative effect of temperature, however, was not significantly different. The responses of *E. coli* to non-lethal temperature were also significantly different between the two types of cheese both for relative and absolute inactivation rates. This was an unexpected result but may have been influenced by the, possibly, anomalous 4°C data for *E. coli* in the blue cheese experiments. Inactivation rates of *L. monocytogenes* in blue cheese were significantly different to that in semi-hard cheese when the 4°C data for semi-hard cheese was included. When the data at 4°C for semi-hard cheese were omitted (see Chapter 3) the response of *L. monocytogenes* in both types of cheese was not significantly different. This result was not unexpected because the $a_w$ and initial pH levels in the blue cheese are very similar to the semi-hard cheese. Inactivation rates of *L. monocytogenes* in blue cheese were significantly different to that in broth (with or without data at 5°C) as was the case in Chapter 3.

Commercially produced Roquefort cheese is ripened for up to one month in caves that are at ~10°C. The experimental cheeses in this work that were incubated at 10°C most closely match the commercial production process proposed by local cheese makers. Maturation at this temperature takes between 3 and 4 months. In the experimental cheese matured at 10°C, *E. coli* was reduced 100,000-10,000,000-fold after approximately 9 weeks. Mould growth was visible after 3 to 4 weeks and there is no evidence of a reduction in inactivation rate corresponding with mould growth or increasing pH (Figure 4.1a iii). In the same cheese, *L. monocytogenes* levels were only reduced by 1,000 - 10,000-fold after 12 weeks (Figure 4.2b iii). The inoculum levels used in these experiments were unrealistically high: natural contamination levels are influenced by the number of animals shedding pathogens and the fact that milk from many animals in a herd is usually mixed together in the bulk milk. High initial inoculum levels were used to demonstrate the extent of microbial inactivation and to allow quantitative evaluation of the inactivation kinetics of microbial pathogens in Roquefort-style cheese (Scott et al. 2005).

It is evident from the results presented that the $a_w$ of the cheese is critical for the prevention of growth of pathogens due to the later increase in pH caused by *P. roqueforti* proteolysis. The critical $a_w$ level required to prevent the growth of *L. monocytogenes* is 0.91 - 0.92 (Ross et al. 2000; Tienungoon et al. 2000). The microbiological quality of milk used for cheese making is also critical. The level of pathogen contamination is of the highest importance because the extent of inactivation of *L. monocytogenes* during normal, commercial, ripening is in the order of 3 to 4 $\log_{10}$CFU as demonstrated in the results presented here and rerported by others (Sanaa et al. 2004). Contamination levels higher than this, however, could allow the survival of pathogenic bacteria. An international expert consultation concluded that relatively high doses are usually required for *L. monocytogenes* to cause human infection (FAO/WHO 2004). The European Union has legislated (EU Regulation 2073/2005 amended by EU Regulation 1441/2007) an upper limit of 100 $cfu.g^{-1}$ at the time of consumption to provide an appropriate level of protection to consumers. Enterohaemorrhagic *E. coli*, is believed to have a much lower infectious dose than *L. monocytogenes* (FDA (United States Food and Drug Administration) 2008). *E. coli* were, however, inactivated almost three times faster than *L. monocytogenes* in the Roquefort-style cheese produced in these experiments. For

a commercial product the time and temperature of maturation is important for the development of flavour in the cheese but also because these factors dictate the extent of inactivation of populations of pathogenic bacteria that may be present in the cheese.

Unpublished data reviewed in FSANZ's assessment report to permit the sale of Roquefort cheese (Anonymous 2005) noted that from challenge studies with *E. coli* O157:H7 inoculated into milk used in Roquefort manufacture none of the *E. coli* could be recovered, even using enrichment techniques at, or after, 70 days of ripening, and that this indicated inactivation "in the order of 5-logs". Those observations support the conclusion from the studies reported here that *E. coli* could be expected to be eliminated during normal processing and ripening of Roquefort. Similarly, other unpublished studies reported by FSANZ (Anonymous 2005) indicate that the rate of inactivation of *L. monocytogenes* was "slower" which is superficially consistent with the observations presented in this work. It is evident from Table 4.2 and Fig. 4.3 that inactivation of *L. monocytogenes* is slower than that of *E. coli*. This raises the issue of whether *L. monocytogenes* contamination levels in milk could exceed the capacity of the ripening process to reduce them to harmless levels. Griffiths (1989) concluded that *L. monocytogenes* cells could survive the process of making raw milk cheeses and remain viable in cheese even after a 60 to 90 day holding period even if levels were as low as $1x10^2$ cfu/ml in milk destined for cheese making. Shroeder et al (2003) reported a case of listerial mastitis in a flock of sheep from which milk was obtained for cheese-making. The average contamination level of *L. monocytogenes* in the milk from infected sheep was $4{\cdot}56 \times 10^4$ cfu/ml and, as noted previously inactivation of *L. monocytogenes* during ripening is in the order of 3 to 4 $\log_{10}$CFU (Sanaa et al. 2004). Concentration (and possibly growth due to delayed fermentation and acidification) was observed during cheese making, resulting in a 60-fold increase in concentration in the cheese, leading to contamination levels in the cheese of up to $3x10^6$ cfu/g. Even after ripening, this could result in levels higher than 100 cfu/g in the cheese at the time of consumption and reinforces the need for assuring the hygienic quality of the milk used in production of raw milk cheeses.

## 4.5 Conclusion

Results presented in this chapter show that the raw ewe's milk Roquefort-style cheese does not support the growth of *L. monocytogenes* nor *E. coli*, and that inactivation of these pathogens occurs under the inimical conditions generated during fermentation and, particularly, maturation. The results show that the inactivation kinetics of *E. coli* and *L. monocytogenes* in raw ewe's milk Roquefort-style cheese are consistent with the hypothesis that vegetative bacterial pathogens in foods that prevent their growth are inactivated at a rate that increases with increasing temperature as hypothesised by Ross et al (2008). Similar observations have been reported for fermented meat products (McQuestin et al. 2006; McQuestin et al. 2009; Zhang et al. 2010) found no systematic difference between the rates of inactivation of *E. coli* and *L. monocytogenes* in inimical broth and hypothesised that the effect of non-lethal temperature on inactivation is not dependent on species. The work presented here on the inactivation of both pathogens in Roquefort-style cheese, and in Chapter 3, shows that that the response of *L. monocytogenes* in an inimical food environment is statistically significantly slower than that of *E. coli.* It remains to be seen if this relationship stands in other food types such as salami or whether it can be repeated in other cheese styles.

The success of the French Government's application to import Roquefort cheese into Australia has increased pressure from interested local parties to manufacture raw milk cheeses domestically. FSANZ identified some gaps in the data presented by French authorities during the review process. This study contributes to and aims to expand the data set pathogen survival during the processing and ripening of Roquefort cheeses made from raw ewe's milk. These results and conclusions are consistent with the FSANZ (Anonymous 2005) risk assessment and ruling that permits the import of French raw ewe's milk Roquefort into Australia provided that milk of good hygienic quality is used and that processes used for Roquefort production are not altered, particularly reductions in time or temperatures. Nonetheless, the results presented here and in previous Chapter offer the possibility of being able to predict the consequences of such changes, and to be able to design alternate processes that confer equivalent microbiological safety.

# Chapter 5 Proteomic analysis of *L. monocytogenes* grown in a model fermentation system

## 5.1 Introduction

Fermentation has long been used as a method of preserving food. Its discovery permitted the preservation of meat, fruit, vegetables and milk for far longer periods than had ever before been possible. Fermentation is an acidification process whereby native flora or added starter cultures ferment carbohydrates to produce organic acids, which serve to enhance the flavour and texture of a food but also act as inhibitors of spoilage and pathogenic bacteria. In raw milk Roquefort cheese, progressive acidification, to below pH 5.0 within 6 to 8 hours and reaching 4.8 within 24 hours, is identified as a key factor ensuring the safety of this cheese type (Anonymous 2005). If *L. monocytogenes* is initially present in raw milk used to make cheese it may survive the fermentation step and go on to be present in the finished product, which is especially hazardous to consumers, and is especially the case for fresh cheeses without extensive salting or extended ripening periods.

As noted in Section 1.3, *L. monocytogenes* is able to invoke an ATR in response to exposure to sub-lethal pH. Thus cells that survive cheese fermentation may invoke an ATR from exposure to conditions encountered prior to or during the cheese fermentation, for example in the bovine udder environment or when ineffective or slow fermentation of milk occurs. Likewise, in cheeses where the pH of fermentation reaches 4.8-5.0, the reduction in pH (and concentration of undissociated organic acids) may not be sufficient to prevent growth but rather may act as a mild acid treatment and induce an ATR. Adaptation during the fermentation process may then provide this pathogen with the means to survive the subsequent salting and ripening processes and thus, upon ingestion, have a greater potential to cause illness.

The development of a stress response by bacteria involves strategies to enhance survival rather than just to maximize growth, and involves multiple genes. Molecular approaches that monitor gene expression, such as DNA microarrays, are useful for providing a whole view of cellular response under stress. Genetic information alone, however, does not provide complete information about biological

processes occurring in response to stress within the cell because the relationship between gene expression (mRNA levels) and gene products (protein levels) do not always correlate (Gygi et al. 1999). A given gene when expressed can be subject to posttranscriptional and posttranslational modification thus expression profiles at the proteome level can potentially provide more useful information about function than profiles at the transcription level (Vaidyanathan and Goodacre 2005; Sonck et al. 2009). Knowing which gene products are expressed and how they come together to constitute a functional unit in response to stress provides relevant information. Accumulation of proteins in a cell at a given time is strongly dependent on the environment experienced by a cell and by its physiological state (Ferguson and Smith 2003). Proteomic studies can thus map cellular proteins in a spatial and temporal manner accounting for post-transcriptional and post-translational changes that are not directly indicated by genome data (Vaidyanathan and Goodacre 2005).

Stress proteins take part in diverse cellular activities including, protein folding, gene regulation, ATP-dependent proteolysis and DNA repair (Chow and Tung 1998; Gu et al. 2000; Krüger et al. 2001; Gustavsson et al. 2002; Rosen and Ron 2002; Miyoshi et al. 2003; Alba and Gross 2004; Van Schaik et al. 2004) and several studies have used proteomic methods to investigate microbial stress responses (Phan-Thanh and Mahouin 1999; Duche et al. 2002; Leverrier et al. 2004; Van Schaik et al. 2004). The process of fermentation encountered by *L. monocytogenes* in cheese may be initially mild enough to act as a sublethal stress allowing cells to synthesise stress proteins and activate protective mechanisms, such as the ATR. It is therefore, of interest to the cheese industry to explore the adaptations that may provide enhanced survival of *L. monocytogenes* to fermentation, rather than just the kinetics of non-thermal inactivation in the system as in the work previously presented. It is likely that *L. monocytogenes* present in raw milk will not survive the ripening period of hard and semi-hard raw milk cheeses, however, survival of the initial fermentation gives rise to the risk of cross-contamination of acid-tolerant *L. monocytogenes* to other cheeses that may allow survival and growth. To better understand the stress response of *L. monocytogenes*, and its potential to survive cheese fermentation, a proteomic analysis of non acid-adapted Scott A cultures, grown under conditions of progressive acidification with lactic acid used to model the process of fermentation, was undertaken. Samples for proteomic analysis, using multidimensional protein

identification technology (MudPIT), were prepared at various stages of the acidification process between the late exponential and stationary phase of growth. Protein expression profiles for biological replicates of strain Scott A were compared to the protein expression profile for strain DS-81, grown to the early stationary growth phase under neutral pH conditions, from work undertaken by Porteus (2008).

## 5.2 Materials and Methods

### 5.2.1 Bacterial Strains and Media

*L. monocytogenes* strain Scott A (ATCC15413, a serotype 4b strain widely used in food-related research) was used to determine protein expression profiles during growth in a slowly acidified broth. The strain was obtained from the School of Agricultural Science Culture Collection (University of Tasmania). *L. monocytogenes* was maintained in nutrient broth (Oxoid CM1, Adelaide, SA, Australia) 30% glycerol (Sigma-Aldrich, Melbourne, VIC, Australia), at -80°C. Cells were recovered on brain heart infusion agar plates (BHA; Oxoid CM225 to which 15g $l^{-1}$ agar was added) by incubation at 37°C for 24h.

### 5.2.2 Growth of *L. monocytogenes* under conditions of decreasing pH

#### *5.2.2.1 Preparation of Stationary Phase Populations of* L. monocytogenes

To prepare experimental inocula, *L. monocytogenes* cells were removed from the surface of thawed stock culture (stored at -80°C) using a sterilised inoculation loop, plated to BHA supplemented with 0.1% sodium pyruvate (Sigma-Aldrich, Castle Hill, Australia) (BHAP) and incubated for 24 (±0.25) hours at 37 (±0.5) °C. Five colonies on BHAP were inoculated to 100 mL Tryptone Soya Broth (Oxoid, CM0131) containing 0.6% yeast extract (Oxoid, CM0019; TSB-Ye) in a 250 mL Erlenmeyer flask and incubated statically at 37(±0.5) °C for 24 (±0.25) hours to achieve a viable count of approximately 9.0 log CFU.$mL^{-1}$. This method was repeated once to produce two biological populations (populations 'A' and 'B').

#### *5.2.2.2 Harvesting* L. monocytogenes *and Inoculation into Test Broths*

Aliquots (1.0 mL) of 24-hour stationary phase populations of *L. monocytogenes* from each of populations A and B were inoculated into approximately 600mL of pre-warmed TSB-Ye in sterile Schott bottle, correspondingly labelled A or B that had been acidified by the addition of 100µL of lactic acid (85%). Each flask contained a sterile magnetic stirrer bar to facilitate rapid dispersal of lactic acid additions. The 600mL broths were incubated in a shaking water bath at 30°C (±0.1). Prior to

acidification and inoculation 4mL of each test broth (A and B) was placed into a sterile L-tube for use as a blank in turbidimetric readings.

#### *5.2.2.3 Preparation of Cell Culture for Proteomic Methods, Enumeration of Viable Cells and Construction of Growth Curves*

One hour after inoculation, test broths were removed from the shaking water bath and placed on a magnetic stirrer plate to maintain culture agitation. Aliquots of 8mL were removed from each of the test broths and placed into sterile L-tubes for turbidimetric analysis and 100-200μL of lactic acid was added aseptically before the flasks were once again placed in the 30°C water bath for an hour. This process occurred each hour over the course of eight hours according to the protocol for acid addition shown in Table 5.1 (*see* Section 5.3), until the pH was reduced to approximately 4.5. The approximate lactic acid concentration reached in broth at pH 4.5 is 23mM in the aqueous phase.

The turbidity of the test broths was monitored hourly just prior to addition of acid. Once transmittance readings dropped by 3-5% the viability of each population was estimated immediately prior to each addition of lactic acid throughout the remainder of the pH treatment. Specifically, 100 μL aliquots were removed and serially diluted in 0.1% bacteriological peptone supplemented with 0.85% NaCl (peptone water). Samples (50μL) were surface plated using a spiral plater (Autoplate 4000 Spiral Biotech Inc., Bethesda USA) onto BHAP. Plates were incubated for 24 (±0.25) hrs at 37 (±0.5) °C and colony forming units (CFU) were counted. Population changes were visualised by plotting $\log_{10}$CFU.mL$^{-1}$ against time. Five samples for proteomic analysis of 10 to 40 mL were taken from each of the test broths at 5, 6, 7, 8 and 24 hours respectively, producing 10 samples at five time points. The samples were placed into 50mL sterile centrifuge tubes and cells were pelleted by centrifugation at 4500 rpm for 10 minutes at 4°C in an Avanti-J-301 centrifuge (Beckman Coulter, USA). The supernatant was poured off and the cell pellets were frozen with liquid nitrogen in preparation for protein extraction.

#### *5.2.2.4 Protein Extraction from Bacterial Cells*

Frozen cell pellets were allowed to thaw on ice for approximately 15 minutes before being washed twice with Phosphate buffered saline (PBS, Oxoid, BR0014G) to remove traces of media and transferred to 1.5mL LoBind Eppendorf® tubes. The soluble protein from each sample was extracted using the Q-Proteome kit (Qiagen) according to the protocol recommended for bacterial cells.

#### *5.2.2.5 Protein Tryptic Digestion*

Sample solubilisation, reduction, alkylation and enzymatic digestion was performed using a protocol developed by Dr. Edwin Lowe (Central Science Laboratory, University of Tasmania, Hobart), adapted from Delahunty and Yates (Delahunty and Yates III 2005). Details of this protocol, including reagents used in the Tryptic digest, are presented in Appendix C (Section C.1).

For sample solubilisation, extracted protein samples were evaporated to dryness using a Mini Ultra Cold vacuum pump (Dynavac, Australia) according to the manufacturer's protocol. Samples were then resuspended in 20µL of 8M urea and 100mM ammonium bicarbonate solution by gentle mixing. Sample alkylation was achieved by adding 5µL of alkylating reagent (200mM iodoacetamide in 100mM ammonium bicarbonate) to the sample, gentle mixing and incubation for one hour at room temperature. Following this, 20µL of reducing agent (50mM dithiothreitol in 100mM ammonium bicarbonate) was added, mixed and left for a further hour. To reduce the urea concentration for enzymatic digestion, 170µL of digestion buffer, 50mM ammonium bicarbonate and 1mM calcium chloride solution, was mixed with the sample. To this, 40µL of trypsin solution 100ng/µL (20µL of Promega sequencing grade modified trypsin in 200µL digest solution) (Sigma, Australia) was added and incubated at 37°C with gentle shaking overnight. The digestion was halted by adding 30µL of 10% formic acid and centrifuging for five minutes at 14000g (Eppendorf® centrifuge 5417R) to remove insoluble material. 200µL of supernatant was pipetted into HPLC sample vials.

*5.2.2.6 LC-MS*

The tryptic digests were analysed using multidimensional protein identification technology (MudPIT) which is a combined liquid chromatography and tandem mass spectrometry (LC/LC-MS/MS) approach with peptide mass spectrometric analysis utilising a ThermoFinnigan LTQ Orbitrap. The system used was a nanoflow triphasic MudPIT system consisting of a C18 capillary trap followed by a strong cation exchange resin (SCX) stage and a final analytical C18 nano-column. The HPLC buffers used are detailed in Table C.1 (Appendix C).

50 µl of tryptic digest was loaded onto the C18 capillary trap at a flow rate of 35 µl/min using Buffer A. During sample loading the SCX and analytical columns were switched via a valve out of line of the C18 trap, with the trap being washed to waste to ensure salts and other non-peptide materials were not introduced into the mass spectrometer. After 10 minutes sample loading the SCX and analytical columns were switched inline with the capillary trap and the flow reduced via a splitter to 250 nl/min. A 5-step MudPIT gradient was performed (described in Appendix B Table B.2). The complete analysis time was 10.5 hours. To ensure no sample contamination occurred between runs, a 1.5 hour clean up method was performed (detailed in Appendix C Table C.3).

The LTQ Orbitrap mass spectrometer is a hybrid mass spectrometer, consisting of a 2D ion trap capable of low resolution MS/MS (LTQ part), and an electrostatic trap capable of performing high resolution, high accuracy, mass analysis (Orbitrap part).

During the MudPIT analysis the mass spectrometer was operated in "data dependent" tandem mass spectrometry (MS/MS) mode. A "survey" scan performed in the electrostatic Orbitrap identified the possible parent peptide masses. The mass spectrometer then made a "data dependent" decision as to what peaks to perform tandem mass spectrometry on, and the top 6 peaks were fragmented in the ion trap, producing MS/MS data. The process takes under 2 seconds to perform and was cycled continually throughout the MudPIT run. A typical LC-MS/MS produced between 10 000 and 20 000 MS/MS spectra to identify. Dr. Edwin Lowe of the CSL (University of Tasmania) processed the samples.

### 5.2.3 Protein Data management

#### *5.2.3.1 Spectral Counting*

Peptide spectra were identified using the X! Tandem search engine with probability of the assessment determined using ProteinProphet probability scoring (Nesvizhskii et al. 2003) based against the derived complete proteomes of sequenced *Listeria* strains, in particular strains EGDe (ATCC BAA-793) and F2365 (ATCC 19115). MS/MS spectral counts for each peptide were pooled for each protein identified from the collective *L. monocytogenes* proteomes. A total spectral count for each protein was then obtained by pooling the values of the two biological replicates analysed for each individual time point. To obtain a sufficient number of peptides for comparison time point samples 5 and 6; and 7 and 8 h were combined. This was done to reduce distortions in the normalization process owing to the large difference in peptide counts that arose from the extraction of different culture volumes (Table 5.1). The pooling was considered acceptable because the time intervals between those pairs of samples was small, and general comparisons of spectral count data showed that the variation between samples at these time points was not substantial.

#### *5.2.3.2 Spectral Count Normalisation*

Normalized spectral counts ($f_1, f_2$) were obtained following the procedure described by Zhang and colleagues ((Zhang et al. 2006)), as follows:

$$f_1 = n_1 \frac{t_2}{t_1} + \lambda, \qquad f_2 = n_2 + \lambda \qquad \text{(Equation 5.1)}$$

where $t_1$ is the total spectral counts for all the proteins in sample 1, $t_2$ is the spectral count of the 24 h sample (Table 5.1); $n_1$ represents the spectral count for a given protein in sample 1 while $n_2$ is the spectral count for a given protein in the 24 h sample; $\lambda$, represents the pseudo spectral count, designated 0.5, used to allow comparison of samples in which a given protein peptide was not detected.

*5.2.3.3 G-test and Fold Change Determinations*

To test that changes that occur in individual protein was significant, the likelihood ratio G-test (Sokal and Rohlf 1995), as adapted for use in label-free proteomic applications (Zhang et al. 2006), was applied:

$$G = 2f_1 \ln\left(\frac{f_1}{\hat{f}_1}\right) + 2f_2 \ln\left(\frac{f_2}{\hat{f}_2}\right) \qquad \text{(Equation 5.2)}$$

where $\hat{f}_1$, $\hat{f}_2$ represent expected spectral counts for the proteins in two samples being compared which are normalized and corrected with the pseudo spectral count correction factor i.e. $f_1$, $f_2$. The null hypothesis assumes that a given protein is equally expressed in two samples, thus $\hat{f}_1 = \hat{f}_2 = (f_1 + f_2)/2$.

To reduce false-positive results occurring due to poorly sampled proteins William's correction ($w$) (Sokal and Rohlf 1995), as adapted for label-free proteomics data by Zhang et al. (2006), was applied:

$$w = 1 + \frac{\left(\sum_{i=1}^{m} \frac{n}{n_i} - 1\right)\left(\frac{n}{x} + \frac{n}{y} - 1\right)}{6n(m-1)} \qquad \text{(Equation 5.3)}$$

$$G_{adj} = G/w.$$

where $n$, is the total spectral count across two samples being compared; $n_i$ are the spectral counts of the separate samples; $x$ is the spectral count of the target protein; and $y$ is the spectral count of the protein in the reference sample. The adjusted G-value ($G_{adj}$) was then compared against a Chi-test distribution table in Microsoft Excel (CHIDIST function) with degrees of freedom equal to 1, in order to obtain a significance value (e.g. G=>3.84, $p$<0.05). To account for type-1 error due to the large number of values being tested in parallel the accumulated significance values

of each comparison was tested for the false discovery rate (FDR) (Benjamini and Hochberg 1995):

$$P_{(k)} \leq \frac{k}{m \cdot c(m)}\alpha \qquad \text{(Equation 5.4)}$$

where $\alpha$ is the significance values being tested; $m$, is the total number of tests (i.e. identified proteins); $c(m)$ is an adjustment value - since the test data can be considered independent, $c(m)$ then equals 1; $k$ is the maximum rank of the significance value in a distribution that can be set for $P(k)$, in this study this was set arbitrarily at 0.01. Fold-change values expressed as a $\log_2$ ratio were obtained by comparing the normalized spectral count values between control samples of strain DS-81 (grown in BHIB, pH 7.3, see Table 5.1) and the samples exposed to the acidic challenge broth. DS-81 was used because there were insufficient resources available to analyse specifically strain Scott A under the same conditions. Strain DS-81 is a factory persistent strain obtained from Rolf Nilsson and, based on peptide profiles and multi-locus sequences typing (MLST), is genetically very similar to Scott A (R. Nilsson, *thesis in preparation*).

#### *5.2.3.4 Protein Classification*

Proteins were classified with functional categories (Table 5.2) according to the scheme belonging to the ListiList database (*genolist.pasteur.fr/ListiList/).* To determine changes in overall relative abundance in proteins within these categories, normalised spectral counts were summed for all proteins within each category. Transporter proteins were classified into three separate sub-categories: phosphotransferase systems, ABC-type transporters, and "other" transporters. Specific proteins central to virulence in *L. monocytogenes* (PrfA, LLO, Mpl, ActA, PlcA, PlcB, InlA, InlB) were assigned to a separate category.

## 5.3 Results

### 5.3.1 Growth of *L. monocytogenes* under conditions of decreasing pH

The experiment used non-acid habituated cultures grown overnight in TSB-Ye at 30°C. Cultures were inoculated into experimental TSB-Ye broths that had been acidified initially by the addition of 100μL of lactic acid to make the broth pH equivalent to that of raw milk used for cheese making. Table 5.1 shows the acidification and sampling protocol for the test broths A and B. The change in numbers for populations A and B is shown in Figure 5.1 along with the evolution of pH for each of the test broths. Arrows mark the time points where samples for proteomic analysis were taken and the amounts of broth culture used are detailed in Table 5.1.

**Table 5.1.** Experimental protocol for the addition of lactic acid to produce the model fermentation broth.

| Sample Time (h) | Amount of Acid Added (μL) to both flasks A and B | pH (average of A and B) | Size of sample for proteomic analysis (mL) | Approximate concentration of lactic acid in the aqueous phase (mM) | Pooled Spectral counts* |
|---|---|---|---|---|---|
| 0 | 100 | 6.97 | - | 1.89 | |
| 1 | 200 | 6.72 | - | 5.66 | |
| 2 | 200 | 6.42 | - | 9.49 | |
| 3 | 200 | 5.76 | - | 13.36 | |
| 4 | 200 | 5.16 | - | 17.38 | |
| 5 | 100 | 4.95 | 10 | 19.27 | 1657** |
| 6 | No acid added | 4.89 | 20 | 19.27 | 4679** |
| 7 | 100 | 4.67 | 40 | 21.37 | 10717*** |
| 8 | 100 | 4.49 | 40 | 23.64 | 9261*** |
| 9 (15 hours later) | No acid added | 4.48 | 40 | 23.64 | 22047 |

*The control is based on strain DS81, which was grown in BHI broth at 25°C for 24 h before harvesting for protein extraction. A total of 4 biological replicates were analysed with a total spectral count of 35627 spectral counts (Porteus 2008). Though not identical to Scott A the strain is genetically similar, based on proteome outcomes and MLST pattern

**Spectral counts at 5 h and 6 h time points were pooled for subsequent G_test and fold change analyses.

*** Spectral counts at 7 h and 8 h time points were pooled for subsequent G_test and fold change analyses.

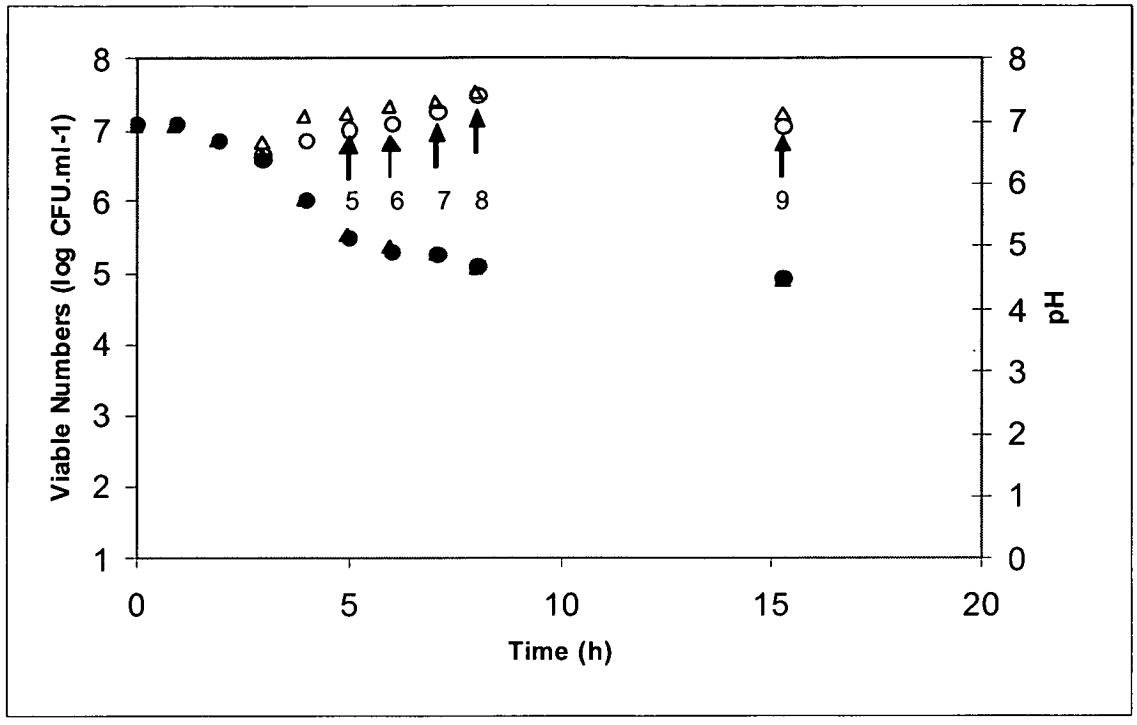


**Figure 5.1.** The change in numbers for populations A (Δ) and B (○) along with the evolution of pH for each of the test broths, A (▲) and B (●). Arrows mark the time points where samples for proteomic analysis were taken.

The initial sample in Table 5.1 was only 10 mL. Consultation with a senior researcher led to the conclusion that larger samples were required to obtain adequate protein expression profiles. The second sample size was increased to 20 mL, however, a further increase in sample volume to 40 mL was implemented to ensure that adequate protein profiles could be obtained. Proteins from each of the time points shown in Table 5.1 were extracted and analysed by the MudPIT technique. Table 5.2 shows the trends in spectral counts within functional protein groups, as assigned by the ListiList database for the acid treated samples and the control.

In comparison with the control, which is in the stationary growth phase, proteins involved with the cell wall, cell sensing, mobility and chemotaxis, phosphotransferase transporters, carbohydrate metabolism, RNA synthesis, elongation and termination, amino-acyl tRNA synthesis, protein elongation and termination, the Sigma B operon and cold shock proteins were all down regulated by varying degrees. The functional groups showing the greatest degree of down regulation are the cold shock proteins, mobility and chemotaxis, and the phosphotransferase transporters.

**Table 5.2** ListiList functional classification codes

| Class Code | Functional classification | control | 5_6 | 7_8 | 9 |
|---|---|---|---|---|---|
| | | Accumulated spectral counts: | | | |
| 1.1 | Cell wall | 657.81 | 553.52 | 530.696 | 526 |
| 1.3 | Sensors (signal transduction) | 293.28 | 284.48 | 241.904 | 242 |
| 1.4 | Membrane bioenergetics | 183.14 | 558.88 | 644.176 | 626 |
| 1.5 | Mobility and chemotaxis | 739.01 | 78.62 | 101.924 | 77.5 |
| 1.6 | Protein secretion | 154.68 | 155.66 | 215.948 | 209.5 |
| 1.7 | Cell division | 145.42 | 580.72 | 507.904 | 440 |
| 1.8/1.9 | Cell surface proteins/internalins | 67.14 | 143.8 | 127.984 | 108 |
| 2.2 | Metabolism of amino acids and related molecules | 733.51 | 645.68 | 1034 | 1394 |
| 2.3 | Metabolism of nucleotides and nucleic acids | 621.82 | 442.16 | 782.408 | 926 |
| 2.4 | Metabolism of lipids | 447.97 | 334.28 | 562.712 | 625 |
| 2.5 | Metabolism of coenzymes and prosthetic groups | 356.55 | 358.22 | 613.748 | 743.5 |
| 2.6 | Metabolism of phosphate | 6.27 | 54.2 | 43.952 | 47 |
| 3.1 | DNA replication | 79.32 | 195.44 | 189.848 | 190 |
| 3.2 | DNA restriction/modification and repair | 172.57 | 327.28 | 361.408 | 382 |
| 3.3 | DNA recombination | 2.61 | 15.92 | 6.416 | 6 |
| 3.4 | DNA packaging and segregation | 206.64 | 527.52 | 300.456 | 279 |
| 3.6 | RNA modification | 271.34 | 260.68 | 472.6 | 498 |
| 3.8 | Protein modification | 103.22 | 114.9 | 148.5 | 128.5 |
| 3.9 | Protein folding | 337.68 | 1655.54 | 1340.924 | 1133.5 |
| 4.1 | Sigma B operon | 62.06 | 24.38 | 38.828 | 37.5 |
| 4.2 | Detoxification and stress defense | 233.28 | 348.12 | 409.608 | 420 |
| 4.3 | Phage-related functions | 30.32 | 402.32 | 313.352 | 206 |
| 4.4 | Transposon and insertional elements | 9.44 | 13.96 | 30.184 | 16 |

| | | | | | |
|---|---|---|---|---|---|
| 4.5 | Miscellaneous | 178.96 | 82.03 | 133.87 | 194.43 |
| 1.1O | Transformation/competence | 6.61 | 19.92 | 21.456 | 17 |
| 1.2.1 | Phosphotransferase transporters | 4820.84 | 624.12 | 546.048 | 559 |
| 1.2.2 | ABC-type transporters | 324.7 | 646.18 | 695.572 | 717.5 |
| 1.2.3 | Other types of transporters | 69 | 156.82 | 154.42 | 106.5 |
| 2.1.1 | Metabolism of carbohydrates and related molecules | 2088.63 | 1665.92 | 1985.984 | 2178 |
| 2.1.2 | Main glycolytic pathways | 2098.92 | 2015.98 | 2095.852 | 2277.5 |
| 3.5.1 | RNA synthesis initiation | 71.1 | 355.48 | 425.728 | 496 |
| 3.5.2 | RNA synthesis regulation | 286.73 | 425.5 | 499.228 | 521.5 |
| 3.5.3/3.5.4 | RNA synthesis elongation and termination | 181.12 | 83.04 | 103.464 | 94 |
| 3.7.1 | Ribosomal proteins | 1248.94 | 4731.46 | 3764.644 | 3201.5 |
| 3.7.2 | Amino-acyl tRNA synthesis | 475.88 | 291.9 | 580.956 | 756.5 |
| 3.7.3 | Protein synthesis initiation | 82.63 | 297.3 | 150.54 | 104.5 |
| 3.7.4 | Protein synthesis elongation | 1185.18 | 1102.68 | 916.008 | 926 |
| 3.7.5 | Protein synthesis termination | 28.23 | 8.96 | 8.624 | 19 |
| 4.1.1 | Cold shock proteins | 1833.33 | 582.66 | 232.236 | 105.5 |
| 5and6 | Unknown and unclassified proteins | 1901.54 | 1986 | 1810.368 | 1595 |
| virulence gene cluster/inlA/inlB | Critical virulence-related proteins | 4.11 | 38.3 | 32.204 | 21.5 |

Some functional groups appear to be down regulated in the combined 5 h/6h and subsequently increase in later samples when compared to the control, including those groups related to metabolism of carbohydrates, RNA synthesis, elongation and termination, and amino-acyl tRNA synthesis. This is also the case with the Sigma B operon, which appears to be initially down regulated when compared with the control; however expression levels do increase over the time course of the experiment. Metabolism of carbohydrates and related molecules initially appears to be down regulated compared to the control but protein levels in this group increase in the experimental samples sequentially. The main glycolytic pathways functional group is stable throughout the experiment and when compared to the control.

The rest of the functional groups listed in Table 5.2 appear to be up-regulated to some extent when compared directly with the control. Significant up-regulation occurs in the functional groups associated with; membrane bioenergetics; protein secretion; cell division; metabolism of amino acids, nucleotides and nucleic acids, coenzymes and prosthetic groups and phosphate; metabolism of lipids; DNA replication, restriction and repair; RNA modification; protein folding; detoxification and stress defense; phage-related functions; ABC-type and other transporters; RNA synthesis initiation and regulation; ribosomal proteins and critical virulence related proteins. In some groups initial protein expression profiles in the 5/6 h sample are higher than those appearing in the same group in the control but over the time course of the experiment the expression levels decrease. These groups include cell surface proteins, metabolism of amino acids, DNA recombination, packaging and segregation, protein folding, other transporters, ribosomal proteins, protein synthesis initiation and critical virulence-related proteins. Slight variations need to be disregarded as the normalisation likely reduces accuracy of the results owing to the under sampling of the 5/6 h sample compared to the other time points.

The protein group 4.11 in Table 5.2 contains proteins with defined or putative involvement in cold stress adaptation mechanisms (cold shock proteins). The general trend in this group is down-regulation over the time course of the experiment. However, a number of proteins found in other groups also have defined or putative actions as cold shock proteins (Tasara and Stephan 2006). Several

specific proteins from other groupings are progressively up-regulated, through the stages of cell growth as shown in Table 5.3.

Three proteins identified as having defined or putative involvement in the cold stress response are down regulated as shown in Table 5.3. In one instance, in the up regulated protein group, two different paralogs of OppA occur and both proteins are coded by different genes e.g. *lmo0152* and *lmo2569* in strain EGDe while in strain F2365 (ATCC 19115) they are coded for by *lmo2365_2225* and *lmof2365_2542.* Another paralog occurs in the down-regulated group of proteins and is coded for by *lmo2196,* as shown in Table 5.3, however levels of OppA (*lmo2196*) are higher after initial exposure to lactic acid than those found in the control and levels decrease over the time of the experiment. The proteins GbuA/ProV, GbuB/ProW, GbuC/ProX, OpuCA, OpuCC, TrxB and OpuCB (up regulated compared to control) are also associated with osmotolerance and the uptake of compatible solutes (Hill et al. 2002). Two cold shock proteins (CspB and CspL), present at higher levels in the control, show decreasing levels of expression at each sample point, however levels are still within 5 per cent of those in the control at the final time point.

Table 5.4 lists genes and their protein products identified as having defined or putative involvement in general stress adaptation and virulence mechanisms from Hain et al (2007) and Tasara and Stephan (2006). The proteins coded for by the sigma B operon, consisting of the genes *rsbR, rsbS, rsbT, rsbU, rsbV, rsbW, rsbX,* are up regulated when considered as a group (Table 5.2), however, interpretation of the expression of proteins coded for by the individual genes within the operon is less clear. The proteins RsbS, RsbU, RsbV, and RsbW are up regulated, RsbT is down regulated and RsbR and RsbX are up regulated only the pooled sample from 7 and 8 hours. Sigma B (coded for by *sigB*) was just detected in the samples in which more severe stress occurs. All other stress related proteins listed in Table 5.4 are down regulated during the acidification treatment except for the proteins Ctc/RplY, ClpB and ClpC (up regulated), and RbfA and LtrC, which become, in general, more abundant with increased lactic acid acidification when active growth is occurring (up to the 7_8 hour samples). This pattern of continued increase for ClpC, ClpB; Ctc, RbfA, LtrC plateaus by 24 hours, indicating that the responses are dynamic and not necessarily linear. In comparison to the control, the heat

**Table 5.3.** Proteins with defined or putative involvement in cold stress adaptation mechanisms in *L. monocytogenes* Scott A exposed to progressive acidification with lactic acid.

| ListiList code | Protein | Function | control | 5_and_6 | 7_and_8 | 9 |
|---|---|---|---|---|---|---|
| | | **Up-Regulated** | | | | |
| | | | Normalized spectral counts*: | | | |
| 2.20 | AroA | phospho-2-dehydro-3-deoxyheptonate aldolase/chorismate mutase | 13.92 | 14.4 | 22.6 | 30.5 |
| 3.7.2 | CysS | cysteinyl-tRNA synthetase | | 3.98 | 10.4 | 16.5 |
| 1.30 | DegU | similar to *B. subtilis* two-component response regulator DegU | | | 7.12 | 12.5 |
| 1.2.2 | GbuA/ProV | putative glycine betaine/proline uptake ABC transporter, ATP-binding protein | | 7.46 | 53.5 | 62.5 |
| 1.2.2 | GbuB/ProW | putative glycine betaine/proline uptake ABC transporter, permease protein | | | 8.23 | 6.5 |
| 1.2.2 | GbuC/ProX | putative glycine betaine/proline uptake ABC transporter, substrate binding protein | | 3.98 | 6.02 | 6.5 |
| 3.5.1 | RpoN | RNA polymerase, sigma-54 (sigma L) subunit | | | 3.81 | 9.5 |
| 2.30 | Prs | phosphoribosyl pyrophosphate synthetase | 13.92 | 35.3 | 43.6 | 44.5 |
| 2.30 | Prs | putative phosphoribosyl pyrophosphate synthetase | 1.72 | | 2.71 | 10.5 |
| 1.2.2 | OppA (*lmo0152*) | putative oligopeptide uptake ABC transporter, substrate binding protein | | 3.98 | 7.12 | 8.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.2.2 | OppA (*lmo2569*) | putative oligopeptide uptake ABC transporter, substrate binding protein | 2.33 | | 3.81 | 8.5 |
| 1.2.2 | OpuCA | putative carnitine uptake ABC transporter, ATP-binding protein | | | 13.7 | 25.5 |
| 1.2.2 | OpuCC | putative carnitine uptake ABC transporter, permease and substrate binding protein | | | | 6.5 |
| 1.40 | TrxB | thioredoxin reductase (NADPH) | | | 10.4 | 17.5 |
| 1.40 | TrxB | thioredoxin reductase (NADPH) | | 63.1 | 118 | 139.5 |
| | | **Down-Regulated** | | | | |
| 1.2.2 | OpuCB | putative carnitine uptake ABC transporter, permease protein | | 3.98 | | |
| 4.1.1 | CspB | similar to cold shock protein; similar to *E. coli* CspC | 336.61 | 56.18 | 17.06 | 15.5 |
| 4.1.1 | CspL | similar to cold shock protein; similar to *E. coli* CspA | 1495 | 522.5 | 214.676 | 89.5 |
| 1.2.2 | OppA (*lmo2196*) | putative oligopeptide uptake ABC transporter, substrate binding protein | 47.47 | 226.7 | 166.1 | 122 |

*Blank values are those in which peptides of the given protein was not sampled. To account for this in the spectral count normalization process a pseudo-spectral count value of 0.5 is applied for these data (see Methods section 5.2.2.8).

**Table 5.4.** Changes in expression of proteins with defined or putative involvement in general stress adaptation, virulence mechanisms and osmotic tolerance in *L. monocytogenes* Scott A exposed to progressive acidification with lactic acid.

| ListiList code | Protein | Function | control | 5_and_6 | 7_and_8 | 9 |
|---|---|---|---|---|---|---|
| | | **General stress proteins** | | | | |
| | | | Normalized spectral counts | | | |
| 3.7.1 | Ctc/RplY | ribosomal protein L25 (general stress protein Ctc) | 22.46 | 35.3 | 76.676 | 76.5 |
| 3.90 | GroEL | class I heat-shock protein (chaperonin) GroEL (Hsp60 complex) | 4.77 | 341.54 | 271 | 213.5 |
| 3.90 | GroES | class I heat-shock protein (chaperonin) GroES (Hsp60 complex) | 18.19 | 254.54 | 151 | 52.5 |
| 3.90 | ClpB | Clp endopeptidase ATP-binding subunit | 14.53 | 7.46 | 48 | 84.5 |
| 3.90 | ClpP | similar to ATP-dependent Clp protease proteolytic component | | 21.38 | 13.7 | 5.5 |
| 3.90 | ClpC | Clp endopeptidase ATP-binding subunit; homologous to *E. coli* ClpB | 10.26 | 38.8 | 96.5 | 118.5 |
| 1.30 | UspA-like | similar to universal stress protein UspA and related nucleotide-binding proteins | 49.91 | 10.94 | 13.7 | 6.5 |
| 1.30 | UspA-like | similar to universal stress protein UspA and related nucleotide-binding proteins | 160.32 | 146.66 | 95.4 | 95.5 |
| 1.30 | UspA-like | similar to universal stress protein UspA and related nucleotide-binding proteins | 11.48 | 3.98 | 1.6 | 3.5 |
| 4.10 | RsbR | positive (anti-anti-sigma B) regulatory factor | 4.16 | | 4.92 | 2.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.10 | RsbS | negative regulator of sigma-B activity (acts against RsbT) | | | | 1.5 |
| 4.10 | RsbT | positive regulator of sigma-B, switch serine/threonine protein kinase | 1.11 | 3.98 | 1.6 | |
| 4.10 | RsbU | phosphoserine phosphatase, regulator of sigma subunit | | 3.98 | | 7.5 |
| 4.10 | RsbV | positive (anti-anti-sigma B) regulatory factor (acts against RsbW) | 23.07 | 3.98 | 8.23 | 7.5 |
| 4.10 | RsbW | sigma-B activity negative regulator; serine-protein kinase | 26.73 | 10.94 | 21.5 | 17.5 |
| 4.10 | RsbX | negative regulator of sigma-B (phosphoserine phosphatase) | 5.99 | | 1.6 | |
| 3.5.1 | SigB | RNA polymerase, sigma-37 (sigma B) subunit | | | 1.6 | 1.5 |
| 3.60 | RbfA | ribosome-binding factor A | 19.41 | | 6.02 | 4.5 |
| 2.40 | LtrC | low temperature requirement C protein; putative phosphatidylglycerophosphatase | 1.11 | | 10.4 | 6.5 |
| 3.90 | GrpE | molecular chaperone (heat shock protein, part of Hsp70 complex) | 2.33 | 80.54 | 57.9 | 32.5 |
| | | **Proteins associated with virulence** | | | | |
| 1.80 | InlA | internalin A | | | 3.81 | |
| 1.80 | InlB | internalin B | | 10.94 | 10.4 | 1.5 |
| 1.80 | InlC | internalin C | | | 2.71 | |
| 1.80 | InlG | internalin G | | | | 1.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.80 | InlH/InlC2 | internalin H | | 3.98 | | 4.5 |
| 1.80 | InlJ | internalin J | 4.16 | | | 1.5 |
| 2.40 | PlcA | phosphatidylinositol-specific phospholipase C | 1.11 | 3.98 | 3.81 | 5.5 |
| 2.40 | PlcB | Zinc dependent phospholipase C | | 3.98 | 2.71 | |
| 4.50 | Hly | listeriolysin O precursor | | 10.94 | 8.23 | 11.5 |
| 3.5.2 | PrfA | listeriolysin positive regulatory protein | | | 1.604 | |
| 1.80 | ActA | actin-assembly inducing protein precursor | | 7.46 | 1.6 | 1.5 |
| | | ***prfA* mediated class 1 stress response genes and protein products** | | | | |
| 3.90 | DnaJ | molecular chaperone (heat shock protein) | | | 7.12 | 18.5 |
| 3.90 | DnaK | class I heat-shock protein (molecular chaperone) | 39.54 | 498.14 | 354 | 325.5 |

stress proteins GroEL and GroES are very high initially in the 5+6 hour samples, which correspond to sample pH between 4.95 and 4.89, and protein levels then decrease in later samples.

Proteins involved in virulence mechanisms are also shown in Table 5.4. The group of peptidoglycan-bound proteins known as the internalins (InlA, InlB, InlC, InlG, InlH and InlJ) show degrees of increased abundance after initial exposure to lactic acid, with levels of expression decreasing as the experiment progressed. Levels of listeriolysin O precursor (*hly)* remain constant throughout the experiment. The protein was not detected in the control. Expression of the protein PrfA was detected once at 7+8 hours and abundance is low, possibly because expression of the protein is induced at 37°C (Johansson et al. 2002), however the *prfA* mediated class I stress response proteins (DnaJ and DnaK) are abundant under acidic conditions. The class I heat-shock protein (DnaK) is found at much greater levels in the sample from 5+6 hours, and at the other time points, than in the control. Expression of the phospholipase PlcA remains stable while PlcB declines after an initial increase in the 5+6 hour samples.

Table 5.5 shows the genes and proteins involved in systems thought to contribute to acid stress adaptation. The first ten listed proteins are subunits that make-up the $F_0F_1$-ATP synthase enzyme that plays a role in the induction of an ATR in *L. monocytogenes* through proton extrusion and ATP synthesis driven by proton motive force, during acid stress (Cotter and Hill 2003). These proteins are classified into the Listilist grouping 1.40, consisting of proteins involved in membrane bioenergetics, which show a group-wide pattern of up regulation (Table 5.2). The majority of the protein subunits are generally up regulated in comparison to the control. Another protein shown to play a role in low pH tolerance of *L. monocytogenes* is the ADI system (Ryan et al. 2009). In this work, only two proteins associated with the ADI system were expressed. ArcA is only seen in the 7+8 hour samples and ArcC appears in only the first two samples. The proteins were not detected in the control. Three glutamate decarboxylases (GadA, GadB and GadB') that make up the Gad system, decline in expression after the 5+6 hour samples. Levels of GadA are higher than the control throughout but levels of GadB and GadB' are always less than those seen for the control. Table 5.6 lists the

prophage-derived proteins classified into the ListiList group 4.30 phage-related functions. As a group the (Table 5.2) these proteins show a significant increase in abundance (when compared to the control) at the 5_6 hour samples with expression levels declining slowly over the time course of the experiment. This response is similar for almost every individual protein listed in Table 5.6 and many of the proteins were not found in the control. A similar pattern of expression has been shown in *E. coli* whereby prophage genes were up regulated after exposure to lactic acid at pH 4.5 (Ramirez Cuevas 2009).

## 5.4 Discussion

The proteomic analysis presented here is biased towards soluble (cytosolic) proteins. Work by Ishihama et al (2008) on the profiling of protein abundance in the cytosol of *E. coli* found that the most abundant proteins samples using a mass spectrometry approach were those found in the soluble portion of the cell. Membrane-bound and peptidoglycan-bound proteins were under-sampled (especially the former). The authors found that the most abundant proteins were the most essential to the cell function, namely ribosomal proteins and those involved in protein synthesis, energy, and proteins with binding functions. Rare proteins were those involved with transcription, transport and cellular organization. In order to sample more of the membrane and cell wall bound proteins a different methodology would need to be used. It would be useful to obtain proteomic information about these peptides in *L. monocytogenes* exposed to lactic acid stress to ascertain if the cell wall and membrane modifications also provide resistance mechanisms to the cell.

Several studies have investigated the effects of strong inorganic (mineral) acids on *L. monocytogenes*, however, less is understood about the tolerance of *L. monocytogenes* to the organic acids routinely used in food preservation to prevent bacterial growth. First documented by Kroll and Pratchet (1992), *L. monocytogenes* can induce an ATR in the presence of mild pH produced by mineral acid (Davis et al. 1996). The ATR induced by exposure to mild mineral acid stress was shown to confer protection against subsequent challenge with acetic acid suggesting the ATR of *L. monocytogenes* may also protect against organic acids (Phan-Thanh and Montagne 1998). Studies by Kroll and Pratchet (1992) together with studies by O'Driscoll, Gahan and Hill (1996) determined that *de novo* protein synthesis is required for the induction of an ATR in *L. moncytogenes*. Leyer and Johnson (1992) showed that acid adaptation promotes survival of salmonellae in cheese so it is conceivable that the same adaptation may also favour the survival of *L. monocytogenes* in cheese.

Organic acids are also known as weak acids and in acidic foods, they are present in both the undissociated (uncharged) and dissociated (charged) forms (Heavin et al. 2009). Organic acids have greater inhibitory effects upon bacterial growth than

inorganic acids due to their characteristic of existing in both the charged and uncharged forms (Vasseur et al. 1999). Undissociated organic acids are thought to act primarily by causing cytoplasmic acidification since the undissociated form of the acid is more able to freely cross the membrane into the more alkaline cytoplasm where it dissociates, releasing a proton, thereby causing a reduction in the pH of the cytoplasm ($pH_i$) (Cotter et al. 1999; Heavin et al. 2009). This mechanism does not appear to be the only way that organic acids affect bacterial growth and in *L. moncytogenes,* growth inhibition by weak acids is also believed to arise because of specific effects of the undissociated acid on cellular physiology, independent of effects on $pH_i$ (Ita and Hutkins 1991; Cotter et al. 1999).

**Table 5.5.** Proteins and genes with defined or putative involvement in general stress adaptation in *L. monocytogenes* Scott A exposed to progressive acidification with lactic acid

| ListiList code | Protein | Function | control | 5_and_6 | 7_and_8 | 9 |
|---|---|---|---|---|---|---|
| | | | Normalized spectral counts | | | |
| 1.40 | AtpA | F0F1-type ATP synthase, alpha subunit | 3.55 | 3.98 | 29.204 | 18.5 |
| 1.40 | AtpC | F0F1-type ATP synthase, epsilon chain | | 3.98 | | |
| 1.40 | AtpC | F0F1-type ATP synthase, epsilon subunit | 44.42 | 146.66 | 105.38 | 74.5 |
| 1.40 | AtpD | F0F1-type ATP synthase, beta subunit | 5.38 | 3.98 | 2.708 | 1.5 |
| 1.40 | AtpD | F0F1-type ATP synthase, beta subunit | | 3.98 | 13.748 | 6.5 |
| 1.40 | AtpE | F0F1-type ATP synthase, subunit c | | 3.98 | 4.916 | 3.5 |
| 1.40 | AtpF | F0F1-type ATP synthase, subunit b | | 21.38 | 17.06 | 28.5 |
| 1.40 | AtpG | F0F1-type ATP synthase, gamma subunit | | | 2.708 | |
| 1.40 | AtpG | F0F1-type ATP synthase, gamma subunit | 5.38 | 66.62 | 54.596 | 69.5 |
| 1.40 | AtpH | F0F1-type ATP synthase, delta subunit | 3.55 | | 4.916 | 7.5 |
| 2.20 | ArcA | arginine deiminase | | | 3.812 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2.20 | ArcC | carbamate kinase | 0.5 | 3.98 | 2.708 | 0.5 |
| 2.20 | GadA | glutamate decarboxylase | 1.11 | 7.46 | 3.81 | 2.5 |
| 2.20 | GadB | glutamate decarboxylase | 45.03 | 28.34 | 25.9 | 21.5 |
| 2.20 | GadB' | similar to glutamate decarboxylase | 5.99 | 3.98 | 2.71 | 1.5 |

**Table 5.6.** Proteins with phage-related functions expressed in *L. monocytogenes* Scott A exposed to progressive acidification with lactic acid.

| ListiList code | Gene name | Function | control | 5_and_6 | 7_and_8 | 9 |
|---|---|---|---|---|---|---|
| | | | Normalized spectral counts | | | |
| 4.30 | | similar to protein gp35 from Bacteriophage A118 | | | 1.604 | |
| 4.30 | | putative phage repressor protein | | | 2.708 | 1.5 |
| 4.30 | LmaA | predicted secreted protein; putative phage tail protein; similar to Antigen A protein | | 84.02 | 32.516 | 15.5 |
| 4.30 | | similar to phage proteins | 1.72 | 0.5 | | |
| 4.30 | | putative bacteriophage tail tape measure proteins | | 3.98 | 1.604 | |
| 4.30 | | similar to protein gp17 from Bacteriophage A118 | | | 1.604 | 2.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.30 | | similar to protein gp18 from Bacteriophage A118 | 1.11 | | 1.604 | 3.5 |
| 4.30 | | unknown protein | | | 2.708 | 1.5 |
| 4.30 | | similar to protein gp20 from Bacteriophage A118 | | | 1.604 | 1.5 |
| 4.30 | AmiA | similar to autolysin (N-acetylmuramoyl-L-alanine amidase I) | | | | 1.5 |
| 4.30 | | similar to phage protein | | | 1.604 | |
| 4.30 | | similar to protein gp30 [Bacteriophage A118] | | | 1.604 | |
| 4.30 | | similar to protein gp23 [Bacteriophage A118] | | | 1.604 | |
| 4.30 | | similar to protein gp21 [Bacteriophage A118] | | 3.98 | 3.812 | 5.5 |
| 4.30 | | similar to protein gp20 [Bacteriophage A118] | 1.72 | | 1.604 | |
| 4.30 | | similar to protein gp18 [Bacteriophage A118] | | | 1.604 | 1.5 |
| 4.30 | | similar to protein gp17 [Bacteriophage A118] | 1.11 | | 3.812 | 1.5 |
| 4.30 | | putative tape-measure; similar to protein gp16 [Bacteriophage A118] | | 10.94 | 13.748 | 3.5 |
| 4.30 | | similar to protein gp15 [Bacteriophage A118] | | | 3.812 | 1.5 |
| 4.30 | | similar to protein gp14 [Bacteriophage A118] | | | 2.708 | |

| | | | | | |
|---|---|---|---|---|---|
| 4.30 | similar to acterial surface proteins containing Ig-like domains; similar to protein gp13 [Bacteriophage A118] | 1.11 | | 2.708 | 1.5 |
| 4.30 | putative major tail shaft protein; similar to protein gp12 [Bacteriophage A118] | | 17.9 | 11.54 | 4.5 |
| 4.30 | similar to protein gp11 [Bacteriophage A118] | | 10.94 | 9.332 | 1.5 |
| 4.30 | similar to protein gp10 [Bacteriophage A118] | | | 1.604 | |
| 4.30 | predicted integral membrane protein; similar to protein gp9 [Bacteriophage A118] | | | 4.916 | 1.5 |
| 4.30 | similar to protein gp8 [Bacteriophage A118] | | 24.86 | 12.644 | 3.5 |
| 4.30 | putative phage coat protein | | 188.42 | 144.02 | 115.5 |
| 4.30 | putative phage scaffolding protein | 1.72 | 7.46 | 7.124 | |
| 4.30 | similar to minor capsid protein; putative portal protein | | 7.46 | 9.332 | 11.5 |
| 4.30 | similar to putative terminase large subunit | | 3.98 | | 1.5 |
| 4.30 | similar to putative terminase small subunit | | | 1.604 | |
| 4.30 | unknown protein | 1.72 | 3.98 | | 2.5 |
| 4.30 | similar to protein gp65 [Bacteriophage A118] | | | 1.604 | |
| 4.30 | similar to bacteriophage tail assembly protein | | | | 2.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.30 | | uncharacterised conserved protein | | | 1.604 | |
| 4.30 | | uncharacterised conserved protein | | | 1.604 | 1.5 |
| 4.30 | | similar to protein gp51 [Bacteriophage A118] | | | 1.604 | |
| 4.30 | Dam | similar to site-specific DNA methylases | 1.11 | | | |
| 4.30 | | similar to putative primosome component and related proteins; similar to protein gp49 [Bacteriophage A118] | | 1.604 | 1.5 | |
| 4.30 | | similar to DNA single-strand annealing proteins | | 10.94 | 2.708 | 1.5 |
| 4.30 | | similar to phage protein | | 3.98 | 4.916 | |
| 4.30 | | similar to protein gp43 [Bacteriophage A118] | | 3.98 | 1.604 | |
| 4.30 | | similar to phage anti-repressor protein | | | 1.604 | 1.5 |
| 4.30 | | unknown protein | | | 3.812 | 1.5 |
| 4.30 | | similar to a putativephage repressor protein | | | | 1.5 |
| 4.30 | | similar to protein gp32 [Bacteriophage A118] | | | | 1.5 |

Lactic acid is the main metabolic product of the fermentation of milk by lactic acid bacteria. It plays a key role in the development of flavour and texture in cheeses but also serves to extend the shelf life and safety of these products (Gravesen et al. 2004). In this study, *L. monocytogenes* Scott A was grown in TSB-Ye that underwent progressive acidification via the step-wise addition of lactic acid in an attempt to mimic the conditions the pathogen may be exposed to in milk undergoing fermentation during cheese production. St-Gelais et al (2009) showed that the evolution of lactic acid in Cheddar cheese reaches at least 1.0 per cent (pH 5.0) at the end of fermentation. Assuming Cheddar has moisture content of 36 per cent, which is common in hard cheeses, (Fox et al. 2000), then the lactic acid concentration can be as high as 110mM in the aqueous phase. The concentration of lactate remains fairly constant in hard cheese except in varieties such as Swiss cheese where secondary bacterial cultures undertake lactate metabolism after approximately 15 to 20 days (McSweeney and Fox 2004). According to the growth model derived from work by Tienungoon et al (2000) the final concentration of lactic acid together with the pH (4.48) reached in broth at the end of this experiment, as shown in Table 5.1, is inhibitory for the growth of *L. moncytogenes*, but lactate levels up to this point are not inhibitory. The cells grew in a broth system that started at pH 6.97 and eventually reached 4.48 after 15 hours. *L. monocytogenes* grew and survived well during the first 8 hours of the experiment and population numbers were only starting to decline by 15 hours (Figure 5.1) leading to the hypothesis that the pathogen may have been able to induce an ATR during the initial mild pH stages of the experiment. Since the ATR is thought to offer cross protection to other stresses the pathogen may encounter during cheese production, such as mild heating and osmotic stress from salting (O'Driscoll et al. 1996; Donnelly 2001), the ability to switch on an ATR in the initial stages of fermentation could prove problematic for producers making raw milk cheese where cheese milk may have a loading of *L. monocytogenes* cells prior to production. Proteomic analysis of cells at various time points throughout the experiment was used to determine whether this was the case.

A number of cellular systems allow *L. monocytogenes* to form an ATR and survive acid stress. The glutamate decarboxylases or GAD system (Cotter et al. 2001) is required to increase tolerance to inorganic acid and to survive the gastric system

(Cotter and Hill 2003). Glutamate decarboxylation (catalysed by either of two carboxylase enzymes, GadA/GadD1 and GadB/GadD2) consumes an intracellular proton contributing to $pH_i$ homeostasis under acidic conditions (Heavin et al. 2009). In the work presented here, three glutamate decarboxylase proteins were quantified (GadA, GadB and GadB') as shown in Table 5.5. Protein abundance levels for all three were down regulated in the samples analysed here, thus the GAD system does not appear to be required for the survival of *L. monocytogenes* under conditions of progressive acidification with lactic acid. These findings are consistent with recent work by Heavin et al (2009) who showed that the GAD systems encoded by *gadA* and *gadB* do not play a role in the ability of *L. monocytogenes* to survive organic acid stress.

The arginine deiminase (ADI) system has also been shown to play a role in the acid tolerance of many bacterial genera including *L. monocytogenes* (Ryan et al. 2009). Ryan et al (2009) recently showed that arginine deiminase, which makes up the ADI system, contribute to low pH tolerance and expression of the ADI pathway is regulated by Sigma B and a dedicated transcriptional regulator, ArgR. The system consists of three enzymes ADI (ArcA), catabolic ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC) and a membrane-bound antiporter (ArcD) (Ryan et al. 2009). ADI catabolises arginine to ornithine, carbon dioxide and ammonia and the ornithine inside the cell is exchanged for a molecule of arginine in an energy-independent manner by ArcD. The ammonia combines with intracellular protons to produce $NH_4^+$ increasing the pH of the cytoplasm (Ryan et al. 2009). However, in this work only ArcA and ArcC were sampled with ArcA found in the 7_8 hour samples and ArcC only found in the first two sampled time points. The low levels and absence in most samples of the ADI proteins would indicate that this pathway either does not play an important role in the ability of *L. monocytogenes* to survive organic acid stress or was potentially under sampled. As with the GAD system, the ADI system may be more applicable to the survival of *L. monocytogenes* to inorganic acid stress as experienced in the GI tract or host phagosomes (Heavin et al. 2009). This conclusion is borne out by the fact that transcriptional analysis of growth and survival was undertaken by Ryan et al (2009) on cells exposed to the inorganic acid HCL.

Sigma B ($\sigma^B$) is an alternative stress sigma factor identified in *L. moncytogenes, B. subtilis* and *S. aureus*. Its function has been compared to that of RpoS/$\sigma^S$ in Gram-negative bacteria (Cotter and Hill 2003). Sigma B has been shown to contribute to tolerance of acid and osmotic stress in *L. monocytogenes* in a number of studies (Wiedmann et al. 1998; Ferreira et al. 2003; Sue et al. 2004; Wemekamp-Kamphuis et al. 2004; Abram et al. 2008a). Transcription of *sigB* is induced by a variety of stress conditions including acidification to pH 5.3 with acetic acid (Becker et al. 1998). In Gram-negative bacteria, the alternative sigma factor RpoS/$\sigma^S$ contributes to the transcription of genes promoting survival under stressful conditions and the expression of virulence genes. Similarly, in *L. monocytogenes* Sigma B has been linked to stress adaptation and virulence (Sue et al. 2004). A number of transcriptional studies have been undertaken on the Sigma B regulon in *L. moncytogenes*. Kazmierczak et al (2003) identified 55 genes in the stationary phase or under conditions of osmotic stress, including several virulence genes, under direct regulation by Sigma B (the Sigma B regulon including *gadB,* the *opuC* operon, *ctc/rplY,* and *rsbV/sigma B* operon genes) providing evidence that Sigma B contributes to stress resistance and to *L. moncytogenes*' ability to infect and survive within a host (Fraser et al. 2003). Whole-genome array analysis of the regulon in *L. moncytogenes* EGD-e revealed 105 Sigma B positively regulated genes and 111 under negative control (Hain et al. 2008). Transcriptomic analyses of the Sigma B regulon in *L. moncytogenes* Scott A have demonstrated that its activity is responsive to osmotic up-shift and temperature down-shift with the greatest response occuring after osmotic up-shift (Wiedmann et al. 1998). Some of these studies have also shown that the Sigma B regulon includes genes encoding virulence factors InlA and InlB (Kazmierczak et al. 2003; Hain et al. 2008) and that Sigma B is required for rapid induction of the expression of genes that may assist *L. moncytogenes* to survive transit through the GI tract. Loss of Sigma B has been shown to result in decreased virulence of *L. moncytogenes* during infection (Nadon et al. 2002; Garner et al. 2006).

The role of Sigma B in surviving weak acid stress has not been explored in detail, although one study suggested that Sigma B does not play a significant role in tolerating acetic acid or lactic acid (Garner et al. 2006). The study by Heavin et al

(2009) which found that the GAD system played no role in organic acid stress response, also found that Sigma B played only a very minor role in protecting cells against weak acids. In this proteomic study Sigma B was only found at low levels of abundance and thus trends in its expression over time cannot be discerned (Table 5.3 General stress proteins). This suggests, like the results of Heavin et al (2009), that its role is minor in the tolerance of *L. monocytogenes* to organic acid stress. The Sigma B operon consists of *sigB* and seven regulators of the *sigB* gene (*rsb*) (Hain et al. 2006). In *B. subtilis* a complex network of proteins coded by the *rsb* genes regulates the activity of Sigma B post-translationally (Hain et al. 2006). The Sigma B operon proteins exhibited dynamic changes in abundance in this experiment. Though abundance levels are low both RsbT and RsbU increased in abundance with the onset of acidic conditions as would be predicted to happen with the onset of energy/environmental stress (Chaturongakul and Boor 2004). RsbV and RsbW act as controllers of SigB levels and their abundance declined initially but then slowly increased again later in the time course (Table 5.4). The results may suggest that initial acidic conditions results in a SigB-dependent response but as lactic acid acidification proceeds the SigB response declines. The work undertaken here, however, was not able to ascertain the specific action that up regulation of both positive and negative Sigma B regulators had upon the expression levels of Sigma B.

The induction of general stress proteins appears to be a non-specific strategy widely used by *L. monocytogenes* to counteract different stressful environmental conditions and may confer cross protection to other stressful environments not yet experienced by the cells (Tasara and Stephan 2006). Induction of the ATR was shown to also protect against thermal and osmotic stress by O'Driscoll et al (1996). The Sigma B regulon includes multiple genes, which contribute to protection against osmotic and cold shock (Kazmierczak et al. 2003). The OpuC operon, the *gbuABC* genes and *oppA* gene code for ABC transporters (Listilist group 1.2.2), which facilitate the uptake of osmoprotectants choline, glycine betaine, and carnitine. Putative Sigma B-dependent promoter sites have been found upstream of the osmolyte transporter genes *gbu* and *opuC* suggesting that components of osmolyte uptake systems in *L. monocytogenes* form part of the Sigma B regulon (Sleator et al. 2003). Protein expression for almost all of these genes is up regulated in this experiment with exposure to lactic acid, as shown in Table 5.3, yet no osmotic stress was introduced

into the experimental broth system during this work. This result indicates, therefore, that the development of an ATR for lactic acid also leads to synthesis of proteins that may provide cross protection to *L. monocytogenes* to osmotic stress, which is of interest in the production of many cheeses which are salted after the fermentation is complete. Two ABC transporter proteins (OpuCA and OppA) are up-regulated by the first time point of the experiment but levels fall in the later stages. OppA is the main oligopeptide transporter that is involved in accumulating peptides as compatible solutes and in recycling cell wall peptides for synthesis of new peptidoglycan (Borezee et al. 2000). Coding of proteins that could provide protection against cold and osmotic stress is very important in systems where *L. monocytogenes* survives the milk fermentation step due to an ATR. It may also survive the salting, drying and refrigeration steps that follow and go on to be present in the cheese at the time of ingestion and potentially cause illness.

Nadon et al (2002) demonstrated that Sigma B influences PrfA, a global regulator of the expression of many virulence genes found in *L. moncytogenes*. PrfA (positive regulatory factor A) is the major regulator of genes making up a virulence gene cluster in *L. moncytogenes*. The PrfA regulon, consisting of 73 genes, includes *prfA, plcA, hly, actA* and *plcB* together with a group of virulence genes that code the internalins (*inlA, inlB* and *inlC*) (Hain et al. 2006). Binding motifs for Sigma B have been observed in the upstream region of 22 PrfA regulated genes and Sigma B has been shown to contribute to infection by *L. monocytogenes* by controlling expression of *inlA* and *inlB* which mediate invasion of *L. monocytogenes* into human intestinal cells (Sleator et al. 2001; Kim et al. 2005). The above-mentioned PrfA mediated virulence genes code for proteins that were up regulated in this experiment except for InlB, PlcB and ActA. Increased abundances of InlB were not large because maximum expression of this protein occurs only at 37°C and may be affected by other factors occurring in an intracellular environment (Johansson et al. 2002). The results from this work indicate that exposure to and survival of acid stress from exposure to lactic acid induces the expression of virulence factors that are required for successful host infection by *L. moncytogenes*. If these cells were to survive cheese manufacturing due to the ATR and cross protection to other stresses, their ability to infect a host could be elevated due to better survival within the gastrointestinal tract and the presence of up regulated virulence factors.

Chaperonins are expressed under stressful conditions and undertake tasks associated with protein folding, protection of denatured proteins and removal of damaged proteins (Hill et al. 2002; Cotter and Hill 2003). DnaKJ chaperones (regulated by PrfA) are up regulated in response to acid stress in many pathogenic bacteria including *L. monocytogenes* (Cotter and Hill 2003). Hanawa et al (1999) showed that DnaK is required for full pathogenesis of *L. moncytogenes*. DnaK is a member of the CIRCE regulon along with GroEL, which also increases in response to acid stress in *L. monocytogenes* and both act as heat shock proteins (Cotter and Hill 2003). GroES is also a chaperone acting as a heat shock protein (Hill et al. 2002). Protein expression levels of chaperonins DnaK and GroES/GroEL (Table 5.4) are elevated greatly compared to the control for samples at 5 hours in this work. Expression levels decrease throughout the remainder of the experiment but remain much higher than the control, even after 18 hours of exposure to lactic acid. The high level of protein expression seen for both of these chaperonins indicates that they could confer protection against heat shock in *L. monocytogenes* exposed to organic acids during fermentation. DnaJ, the DnaK co-chaperone protein also has increased abundance consistent with the observations for DnaK. PrfA also regulates the Clp proteases, coded for by the ClpC operon, which are also involved in heat tolerance and virulence in *L. monocytogenes* (Hain et al. 2007). In particular, ClpP is though to be required for the full activity of the haemolysin, Listeriolysin O (LLO), coded for by *hly* (part of the PrfA regulon) (Hill et al. 2002). LLO is essential to allow bacterial escape from phagosomes and optimal haemolytic activity occurs at acidic pH (Kayal and Charbit 2006). LLO, ClpP, ClpB and ClpC all occur at much higher abundance than in the control.

Proton pumps are another method used by many bacteria to resist low pH. The $F_0F_1$-ATPase consists of a multi-subunit enzyme which consists of a catalytic portion ($F_1$) (with $\alpha, \beta, \gamma, \delta, \varepsilon$ subunits) which can synthesis or hydrolyse ATP and an integral membrane portion ($F_0$) (with *a, b* and *c* subunits) which acts as a membrane channel for proton dislocation (Hill et al. 2002). Datta and Benjamin (1997) confirmed the role of the $F_0F_1$-ATPase in the resistance of *L. monocytogenes* to acid stress. Cotter et al (2001) confirmed that the $F_0F_1$-ATPase also plays a role in the induction of an ATR in *L. moncytogenes*. The *b* subunit of the ATPase found in *L. monocytogenes* is induced by acid stress and the genes coding for the $\varepsilon$ and $\beta$ subunits, *atpC* and *atpD*,

are preceded by promoter boxes associated with the positive virulence gene regulator PrfA (Cotter and Hill 2003). In this work, proteins classified into Listilist group 1.40 (membrane bioenergetics), which includes $F_0F_1$-ATPase subunits, are up regulated up to >60-fold (Table 5.5). Up regulation is apparent for AtpH, AtpG, AtpF, AtpeE, AtpD and AtpA. The ε subunit (AtpC) shows high initial protein expression levels compared with the control and levels slowly decrease throughout the remainder of the time course of the experiment.

Lysogeny is the fusion of the nucleic acid of a bacteriophage with that of a host bacterium and it is widespread among *Listeria* strains. The newly integrated genetic material is called a prophage and of these many are cryptic, and it is unclear how they affect the phenotype of the host cell, however in a stressful environment where DNA host damage is likely to result in cell death the proportion of prophage excision and lysis increases (Loessner et al. 2000). Later stress exposure (i.e. exposure to UV radiation) can cause lysis and release of new phages. Lysogenic conversion may also occur when temperate phages induce changes in the bacterial phenotype such as enhanced virulence and genes present in prophages without a clear phage function may code for proteins that act as fitness factors enabling the host to survive adverse environmental conditions (Faruque et al. 2004; Hermans 2007; Garcia-Russell et al. 2009). The phage-related protein group (ListiList group 4.3), Table 5.2, shows high abundance levels after exposure to lactic acid with abundance decreasing moderately throughout the rest of the experiment. Phage-related proteins are present at low levels in the control cells. A similar phenomenon in microarray data was found by Ramirez Cuevas (2009) in *E. coli* OH157:H7 strain Sakei treated with lactic acid at pH 4.5. This finding is interesting as it appears common to both a Gram-positive and a Gram-negative species exposed to similar environmental conditions. It is hypothesised that the phage-related proteins expressed under organic acid stress may play a role in possible survival mechanisms (Ramirez Cuevas 2009). Further work with microarray analysis of *L. monocytogenes* and proteomic analysis in *E. coli* under the same conditions would assess the reproducibility of his phenomenon and lead to possible reasons why this occurs.

The data discussed in this work is by no means final. Many other proteins, not directly involved with an ATR, showed substantial expression and dynamic change

throughout the experiment and analysis of those data is of interest for future work (raw data available on CD at the end of this publication). In all, the data presented here show the large amount of useful information that is obtained from proteomic analyses, especially now that whole genome sequences for multiple *L. monocytogenes* strains have become available. The results could have been enhanced, however, through the use of control data from the same strain and at the same stage of growth. In the work presented here, control data came from cells of strain DS81 grown at 25°C to early stationary phase before protein was extracted. This experiment analysed protein expression for actively growing cells of strain Scott A at 30°C. Of interest for further work is to compare this experimental data to protein expression levels for Scott A cells allowed to grow at 30°C under condition of normal pH with samples taken at the same intervals as for this work to allow for direct comparison of protein expression levels. Another factor that may have introduced a level of error is the fact sample sizes throughout the time course of the experiment were not consistent. This work, however, has identified relevant molecular elements central to the ability for *L. monocytogenes* to tolerate stress that with additional research will further illuminate mechanisms related to survival of organic acids encountered during food fermentation. One other avenue of further work, should the resources be available, is a comparison of Scott A cells in the model fermentation broth at the temperatures examined in the kinetics of non-thermal inactivation chapters and to follow the experiment to its natural end obtaining samples not only during active growth but also during inactivation. Comparison of protein expression profiles at 30°C with the profiles of cells at lower temperatures may provide an insight into the mechanisms of non-thermal inactivation in *L. moncytogenes*.

## 5.5 Conclusion

In foods such as cheese, bacterial pathogens must overcome a series of hurdles such as low pH in order to survive and go on to cause food-borne illness. Many organisms are able to produce an ATR, in which exposure to mild pH allows cells to become resistant to much lower pH and in some cases to stresses other than that used to induce the ATR. *L. monocytogenes* is one organism where the ATR is well documented (Kroll and Patchett 1992; Gahan et al. 1996; Hill et al. 2002; Cotter and

Hill 2003). Understanding how *L. monocytogenes* behaves in the presence of lactic acid and the factors that produce the ATR in foods is vital. The ability of *L. monocytogenes* to survive the sub-optimal conditions it encounters in foods may favour its survival during transit through the gastrointestinal tract and entry into host cell phagosomes and subsequent proliferation (Becker et al. 1998) Most proteins designated as virulence factors are proteins that respond to stressful conditions and assist the organism to survive these. Stress conditions encountered in foods, such as high osmolarity and exposure to organic acids, enhance the ability of *L. monocytogenes* to invade human epithelial cells (Garner et al. 2006; Abram et al. 2008b). Expanding our knowledge about the methods pathogens use to protect themselves against stresses encountered in food production systems may also assist in the optimization of current processes and the development of new efficient methods of food preservation, e.g. optimization of non-thermal death processes. In the production of cheese a fermentation that fails to proceed at the correct speed or fails to reach a goal pH may select for *L. monocytogenes* cells that could go on to survive salting and ripening processes and cause illness. This work has shown that it is vital for cheese production systems, especially those using raw milk, to ensure adequate fermentation occurs and that subsequent stages of salting and drying are also of a severe enough nature to inhibit pathogenic bacterial growth to produce a safe ready to eat food.

## Chapter 6 Thesis Summary

The primary objective of this study was to expand understanding of the effects of temperature, low pH and $a_w$ on the kinetics, and mechanisms of inactivation of *E. coli* and *L. monocytogenes* in raw and pasteurised milk cheeses. Initially the kinetics of non-thermal inactivation of *L. monocytogenes* in an inimical broth environment were described. Later investigations provided further information about the kinetics of non-thermal inactivation of *L. monocytogenes* and *E. coli* in two styles of fermented cheese. A final investigation provided insight into the mechanisms responsible for, and the physiological response of, cells able to survive during lactic acid fermentation.

The inactivation kinetics of *L. monocytogenes* in laboratory broth at pH 3.5 and $a_w$ 0.90 (Chapter 2) generally supported the hypothesis of Ross et al (2008) that temperature within the non-thermal range is the main factor governing the inactivation of *L. monocytogenes* when cells are prevented from growth by other environmental factors. The results of the broth studies of inactivation kinetics were not significantly different to those of Zhang et al (2010) but only if low temperature data from the current work were removed from the analysis. The inactivation rate determined at 5°C appeared to be anomalously fast when compared to the inactivation rates from Zhang et al (2010) whose work included one strain in common with this study. Upward concave curvature was observed in the Arrhenius plot of *L. monocytogenes* responses in inimical broth, but was not observed by Zhang et al (2010) in *L. monocytogenes* and *E. coli*. A quadratic better described the response than a linear model when the 5°C data were included but not when they were removed. It appeared that the 5°C data were anomalous and may have been caused by some equipment problems causing variation in experimental temperature as discussed in Section 2.4. Investigations described in Chapter 2 also indicated a strain effect on inactivation kinetics. This observation requires further investigation but, in the case of *L. moncytogenes,* two strains known to have greater than average acid resistance, demonstrated longer inactivation times under inimical conditions in laboratory broth. Differences in strain responses were a large source of variation but this was not explored in further detail due to time constraints. The evidence from the work in Chapter 2 is that strain differences can have as great an effect on inactivation

rate as a 5 – 10°C shift in temperature. This has consequences for studies based on cocktails of strains and might play some part in explaining the curvature observed in the inactivation rates of *L. moncytogenes*. Thus, strain choice should be considered for all further inactivation kinetics work.

A review of published inactivation data for *L. monocytogenes* in foods and analogous aqueous broth systems was undertaken in Chapter 2 to further evaluate the hypothesis of Ross et al (2008) and observations of McQuestin et al (2009). A meta-analysis of *E. coli* inactivation in fermented meats by McQuestin et al (2009) found temperature dominated the rates of inactivation of *E. coli* in inimical food and analogous broth systems. The Arrhenius model used in this study (Figure 2.9) did not explain the variance in the inactivation rates as well as found for *E. coli* by McQuestin et al (2009). The meta-analysis undertaken in this study used inactivation rates from a much larger range of food environments than the work of McQuestin et al (2009). It also initially used modelled datasets and others that were not published in peer reviewed publications. Analysis of data from the peer reviewed literature only (*sans* modelled datasets) revealed a greater explanatory effect of temperature upon the inactivation rate of *L. moncytogenes*. The meta-analysis also found that $a_w$ had a pronounced effect upon the under- or over-prediction of the Arrhenius model used to describe the effect of temperature. Many of the studies used, however, did not provide $a_w$ information, therefore, only a small proportion of the whole data set was able to be used for the residuals analysis (Figure 2.10), which may have 'skewed' the results in favour of a large $a_w$ effect. The *L. monocytogenes* data used in the meta-analysis also showed the upward tailing curvature that was observed in the broth data in this chapter. It is possible that the response of *L. monocytogenes* to inimical conditions at low temperature is affected by factors other than just temperature, but it was not possible to determine or infer, those factors, from the studies and analyses undertaken as part of this thesis. Raw data from the meta-analysis by McQuestin et al (2009) was not available at the time of writing, however, to conclusively determine whether the effect of non-thermal temperature on *E. coli* was, indeed, similar. A detailed statistical analysis of the two datasets is required, and forms the basis for future work. If the effect of non-thermal temperature is, indeed, non-species specific it would provide a simplifying principle for assessing safety of non-heated food products.

The results presented in Chapter 2 provided some support for the non-thermal temperature inactivation hypothesis. This led to expanding the dataset with inactivation data in a fermented cheese the pH and $a_w$ of which could, like salami, become inimical to pathogenic microbial contaminants. The semi-hard cheeses assessed (Chapter 3) did not allow the growth of *E. coli* or *L. monocytogenes* and showed a temperature-dominated pattern of inactivation for both species. The first apparent conclusion from the studies of microbial inactivation in semi-hard cheese is that inactivation of *E. coli* is significantly different in its relative response to temperature than inactivation of *L. moncytogenes.* *E. coli* inactivation is also significantly faster. This result does not agree with that of Zhang et al (2010) who concluded that there was no significant difference in the inactivation rates of either species in inimical broth. The rates of inactivation in semi-hard cheese are also significantly different to those of the same organisms in broth. McQuestin et al (2009) also noted that inactivation in broth was faster that inactivation in foods for *E. coli*, but the Arrhenius models used to describe the response of the organisms to temperature also have different slopes to the models for broth inactivation. From visual examination of Figure 3.5 it also becomes more apparent that the inactivation response of *E. coli* in cheese is different to that in a salami product reported by McQuestin (2006). Again access to the raw data was not available for statistical analysis of the degree of difference. An upward curvature in the *ln*(inactivation rate) data for *L. monocytogenes* was again observed suggesting that this phenomenon is unlikely to be experimental artifact but rather a real phenomenon. The cheese system used in Chapter 3 involved experiments in a medium with many uncontrolled variables, including changing pH and $a_w$, lactic acid starter bacteria and a complex matrix of protein and fat which may have some influence (possibly protective) upon the temperature response of *E. coli* and *L. moncytogenes*. The hypothesis of Ross et al (2008) is not refuted but the work in Chapter 3 shows that the hypothesis may not hold for pathogen inactivation in complex food products.

In Chapter 4 studies involving a more complex cheese product to investigate inactivation kinetics of *L. monocytogenes* and *E. coli* were described. The cheese was one in which a secondary blue mould culture was encouraged to grow, and which caused the cheese to have fluctuating pH profiles. Time-based pH and $a_w$ data were obtained for this series of inactivation studies in raw ewe's milk cheese to

enable a more thorough assessment of the non-thermal temperature inactivation hypothesis. The pH of the blue cheese approached pH 7 during the ripening phase (Figure 4.4) but there was no evidence that the changing pH affected the inactivation of either *E. coli* or *L. moncytogenes*. As with the responses described in Chapter 3, the inactivation of *L. monocytogenes* was significantly slower in blue cheese than the inactivation of *E. coli*. The relative effects of temperature, however, were not significantly different, unlike the results for semi-hard cheese presented in Chapter 3. Both *E. coli* and *L. monocytogenes* exhibited upward tailing curvature in the *ln*-transformed inactivation rate data and both data sets were statistically better described by a (more complex) quadratic model. Given the repeated observation of this phenomenon it is most likely to be genuine, i.e. not an experimental artifact, in *L. monocytogenes* but it has not been observed in other experiments in this study for *E. coli* or in the work of McQuestin (2006), McQuestin et al (2009) or Zhang et al (2010). However, since the raw data from McQuestin et al (2009) are not available it cannot conclusively be said that the meta-analysis of *E. coli* inactivation is not also better explained by a more complex model than the Arrhenius model. That is not to say, however, that it isn't a reproducible phenomenon. In the case of *E. coli* the data set in which 'tailing' was observed may be an experimental artifact caused, e.g., due to inoculum preparation, or because in the blue cheese *E. coli* was inoculated into the same cheese as *L. moncytogenes*, unlike the studies described in Chapter 3 in which individual cheeses were inoculated with only one species of pathogen. The inactivation of *E. coli* was significantly slower in the blue cheese than in the broth work of Zhang et al (2010). This was also the case for inactivation of *L. monocytogenes* in blue cheese compared to broth (Chapter 2) but the inactivation of *L. monocytogenes* was also systematically different in blue cheese (and semi-hard cheese) than in broth. The initial hypothesis by Ross et al (2008), i.e., that there was no species difference in the inactivation response by temperature was supported by Zhang et al (2010) for a narrow range of inimical conditions. However the challenge studies in cheese do refute the hypothesis, consistently showing that *E. coli* inactivates more rapidly in cheese than *L. moncytogenes*. There could be many physiological reasons for the systematic difference such as cell wall differences or the different range of optimal growth temperatures for those species. It was hypothesised that since both species have been observed to induct an ATR that some

of the differences seen in inactivation rates in fermented foods could be attributable to this phenomenon.

For the proteomic analysis of the ATR in pathogenic bacteria exposed to fermentation, a model broth system was devised to mimic the change in pH during the first 24 hours of cheese fermentation. Actively growing *L. monocytogenes* cells exposed to the model fermentation system were found to up-regulate expression of numerous stress response and virulence proteins. Many of the proteins expressed are not directly involved in acid tolerance but are involved in the heat shock and osmotic shock systems of *L. moncytogenes,* potentially conferring cross protection to many of the stresses that acid tolerant cells in milk and cheese may encounter further along during the manufacture of cheese. The studies described in Chapter 5 were a first step in characterizing the proteomic response of pathogenic cells in fermented food products. Further work is required to detail the responses of both *L. monocytogenes* and *E. coli* to fermentation systems at a low (5°C) and intermediate (15°C) temperature like those tested in the broth and cheese work which would further aide our understanding of the varied responses observed in the inactivation rates of these organisms in foods and broths. The slower inactivation rates observed in *L. monocytogenes* may well be affected by the ATR but *E. coli* is also known to induce an ATR. Future investigation would enable confirmation of whether the ATR under fermentation conditions in both species is similar and whether it can account for the (sometimes) erratic inactivation response observed and described earlier.

The results presented in this thesis offer the possibility of being able to predict the consequences of manipulating manufacturing conditions for ready to eat foods in order to design processes that confer microbiological safety. The results from this work reinforce the slower inactivation of *L. monocytogenes* than *E. coli* in both raw and pasteurised cheeses, and raise the issue of whether *L. monocytogenes* contamination levels in milk used for cheese making could exceed the capacity of the ripening process to otherwise reduce them to harmless levels, emphasising the need for raw milk to be of good microbiological quality whether it is heat treated or used raw.

In conclusion, this thesis has contributed to the knowledge and understanding of the non-thermal temperature induced inactivation of *L. monocytogenes* and *E. coli* under inimical conditions. The results of this study refute the finding and hypothesis of other authors with regards to the absolute rates of non-thermal inactivation of pathogenic bacteria in ready-to-eat food products being unaffected by bacterial species. However, the relative effect of temperature is statistically consistent across the species and environments tested here, with the exception of the results from Chapter 3, and with the work of other authors. Once inimical conditions are met, temperature is the dominant factor affecting the rate of inactivation even when conditions such as pH change (Chapter 4). The work presented here has also indicated avenues for further studies to reveal the mechanisms of non-thermal inactivation of pathogenic bacteria in inimical systems.

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## Appendices

## Appendix A: Materials and Equipment

### A.1 Materials

### A.1.1 Bacterial Strains

*L. monocytogenes* and *E. coli* strains used in this thesis are described in Table A.1. Colonies taken from original plate cultures were resuspended in Nutrient Broth containing 30% glycerol and duplicate stocks stored at -80°C. One culture was used for the preparation of experimental inocula while the other was held in reserve. To prepare experimental inocula, cells were removed from the surface of the thawed stock culture using a sterile yellow pipette tip, plated to BHA and incubated for 14 hours (*E. coli*) or 24 hours (*L. monocytogenes*) at 37°C. All strains were sourced from the School of Agricultural Science Culture Collection (University of Tasmania) except strain R31 which was sourced from the Clinical School of the University of Tasmania.

Table A.1. *L. monocytogenes* and *E. coli* strains used in studies described in this thesis.

| *Strain* | *Description* |
|---|---|
| *L. monocytogenes* | |
| ATCC 19115/Li 2 | Clinical (cerebrospinal fluid) isolate serotype 4b |
| LO28 | Clinical isolate serotype 1/2A |
| 70-1700 | Sheep isolate serotype 4e |
| Scott A | Pathogenic type strain serotype 4b |
| FW03-0035 | Processed meat product isolate serotyope 4b |
| 79-2759 | Isolated from sheep's milk serotype 4a |
| 79-0430 | Isolated from sheep's milk serotype 4a |
| *E. coli* | |
| M23 | OR:H-, acid tolerant, *non-* |

| | |
|---|---|
| | pathogenic strain |
| MG1655 | K12 derivative, *non*-pathogenic |
| R31 | Verotoxigenic clinical non-typable isolate |

### A.1.2 Media and Reagents

#### *Culture-based Studies*

Bacteriological peptone, yeast extract and all broth culture media were obtained from Oxoid (Australia). Agar (Grade J3) was supplied by Gelita (Australia). BDH (Australia) supplied the standard, reference, pH solutions. Reagents were obtained from Sigma (USA) included glycerol (~99% purity), and pyruvate (sodium salt, >99% purity). Sodium chloride (99% purity) was supplied by Chem-supply (Australia) and hydrochloric acid (31.5% purity), lactic acid (85% purity) and tri-sodium citrate (sodium salt, 99% purity) were supplied by Ajax Chemicals (Australia). Antifreeze (polyethylene glycol) was purchased from BP (Australia).

#### *Proteomic studies*

The Q-Proteome kit was manufactured by Qiagen (United States of America) and Promega sequencing grade modified trypsin was purchased from Sigma (Australia).

### A.1.3 Bacteriological Media

The bacteriological media used throughout the course of this study were prepared and stored according to the manufacturer's instructions (Oxoid, www.oxoid.com), or as described below.

#### *Brain Heart Infusion Agar with 0.1% sodium pyruvate (BHAP)*

| | |
|---|---|
| Brain Heart Infusion broth (Oxoid CM0225B) | 37g |
| Agar (1.5%) | 15g |
| Sodium pyruvate (0.1%) | 1g |
| $dH_2O$ | 1L |

Autoclaved (121°C, 20 minutes).

***Tryptone Soya Agar 0.6% yeast extract (TSA-Ye)***

| | | |
|---|---|---|
| Tryptone Soya broth | (Oxoid, CM0129B) | 30g |
| Agar (1.5%) | | 15g |
| Yeast extract (0.6%) | | 6g |
| $dH_2O$ | | 1L |

Autoclaved (121°C, 20 minutes).

***Tryptone Soya Broth 0.6% yeast extract (TSB-Ye)***

| | | |
|---|---|---|
| Tryptone Soya broth | (Oxoid, CM0129B) | 30g |
| Yeast extract (0.6%) | | 6g |
| $dH_2O$ | | 1L |

Autoclaved (121°C, 20 minutes).

***Nutrient Broth (NB) ± 30% glycerol***

| | | |
|---|---|---|
| Nutrient broth | (Oxoid, CM0001B) | 13g |
| $dH_2O$ | | 1L |

Autoclaved (121°C, 20 minutes). When required, 300ml glycerol was added prior to the autoclaving to give nutrient broth with 30% glycerol.

### A.1.4 Solutions

Solutions were stored at room temperature unless otherwise stated.

***Standard diluent***

| | |
|---|---|
| Bacteriological peptone (0.1%) | 1g |
| NaCl (0.85%) | 8.5g |
| $dH_2O$ | 1L |

Autoclaved (121°C, 20 minutes).

***Tri-sodium citrate solution***

| | |
|---|---|
| Tri-sodium citrate | 20g |
| $dH_2O$ | 1L |

Autoclaved (121°C, 20 minutes).

***Cheese diluent***

| | |
|---|---|
| Standard diluent | 400ml |
| Tri-sodium citrate solution | 500ml |

Pre-warmed in water bath to 40-45°C.

## A.2 Equipment

***Autoclave***

Pressure cooker RY-150 from Rinnai (Australia) used with countdown timing systems.

***Centrifuges***

1. Microcentrifuge 5417R, Eppendorf, Germany
2. Universal 16A, Imbros, Australia
3. Avanti-J-301 centrifuge, Beckman Coulter, Australia

***Freezer (-80°C)***

Ultra-low temperature freezer MDF-U50V, Sanyo, Japan

***Incubators***

1. Water bath (model SWB20D), Ratek Instruments, Australia used with a refrigeration unit (Model RC2), Ratek Instruments, Australia. An antifreeze solution (1:1 with $dH_2O$) was used in this water bath, which was accurate to within ± 0.1°C of the set temperature. Incubations were static or with shaking as required.
2. PsycroTherm Controlled Environment Incubator Shaker, New Brunswick Scientific, USA. Incubations were conducted without shaking.
3. Qualtex incubator, Watson Victor Ltd., Australia

4. Refrigerated incubator, Axyos, Australia

***Magnetic stirrer***

Series 5Q0, Activon, Australia

***pH meter***

Model 250A, Orion Research Inc. (USA) fitted with Activon AEP flat tip probe from Activon Scientific Products Co. Pty. Ltd., Australia. The instrument was calibrated before use according to the manufacturer's instructions with standard pH solutions (pH 7.00 and pH 4.01).

***Pipettes***

Various Gilson Pipetman pipettors, John Morris Scientific, Australia. Volumes were calibrated annually by weighing the dispensed volume of $dH_2O$ at RT to ± 1% of the set volume.

***Spectrophotometer***

SmartSpec™ 3000, BioRad, USA.

***Spiral plater***

Autoplate 4000 , Spiral Biotech Inc., USA.

***Stomacher***

Colworth 400, A.J. Seward, UK.

***Temperature data logger***

Tinytag®, Hastings Data Loggers, Australia.

***Vacuum packer***

Technovac T60 vacuum packer, San Paolo, Italy.

***Water activity meter***

Aqualab CX-2, Decagon Devises, USA. Calibrated before use according to the manufacturer's instructions.

## Appendix B: Inactivation Experiments

### B.1 Inactivation curves for *L. monocytogenes* strains LO28 and 70-1700 at pH 3.5 and $a_w$ 0.90.

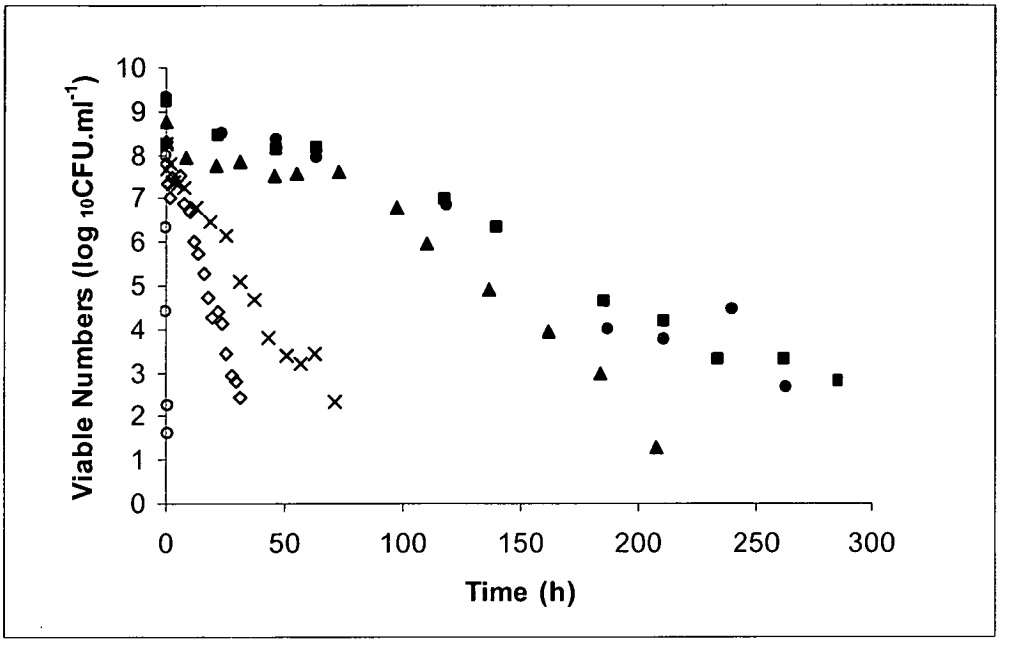


**Figure B.1.** The inactivation curves for *L. monocytogenes* LO28 suspended in broth with pH 3.5 (HCl as acidulant) and $a_w$ 0.90 (NaCl as humectant) and incubated at 5°C (•), 10°C (■), 15°C (▲), 25°C (×), 35°C (◊), and 45°C (○).

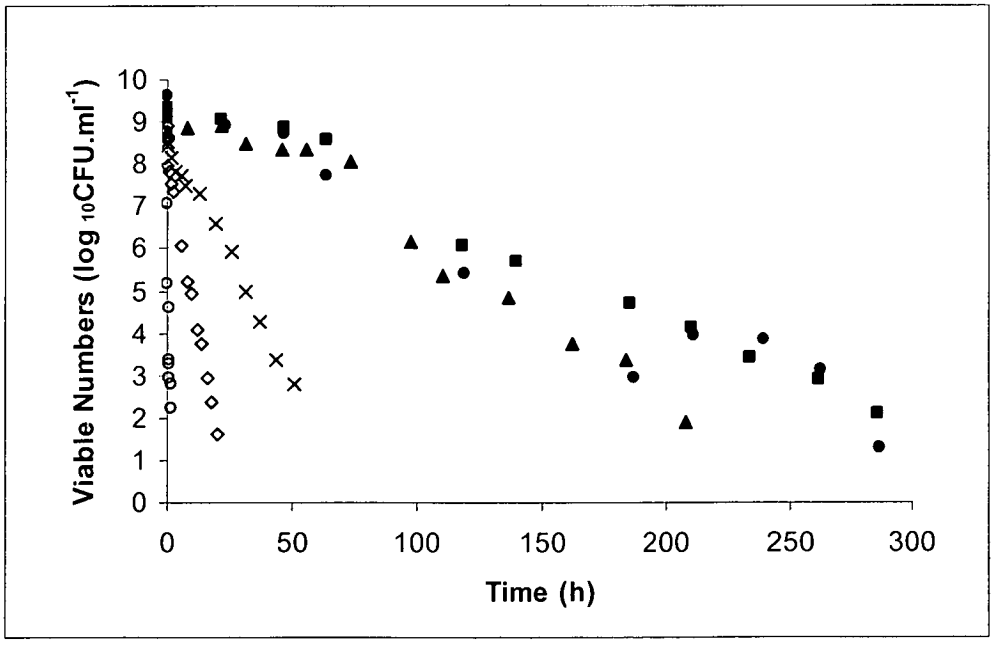


**Figure B.2.** The inactivation curves for *L. monocytogenes* 70-1700 suspended in broth with pH 3.5 (HCl as acidulant) and $a_w$ 0.90 (NaCl as humectant) and incubated at 5°C (•), 10°C (■), 15°C (▲), 25°C (×), 35°C (◊), and 45°C (○).

## B.2 Non-thermal inactivation curves for *L. monocytogenes* at pH 3.5 and $a_w$ 0.90.

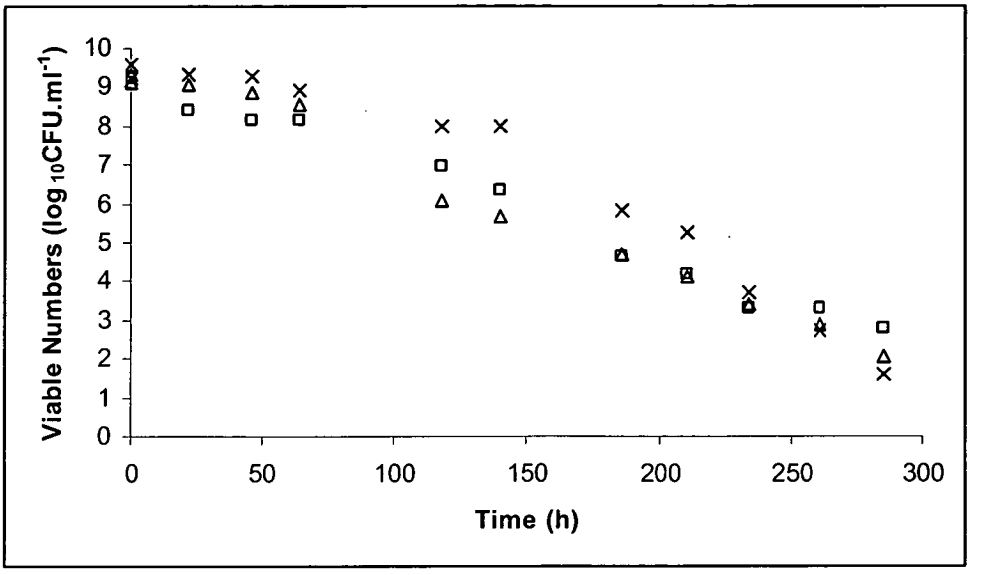


**Figure B.3.** Representative non-thermal inactivation curves for *L. monocytogenes* ATCC 19115 (×), LO28 (□), and 70-1700 (Δ) suspended in broth with pH 3.5 (HCl as acidulant) and $a_w$ 0.90 (NaCl as humectant) and incubated at (a) 10°C.

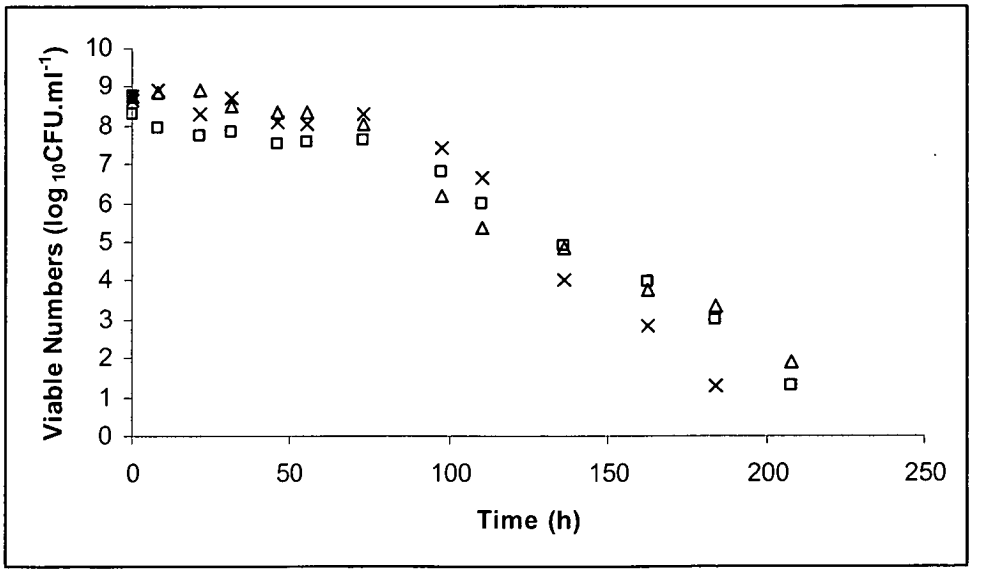


**Figure B.4.** Representative non-thermal inactivation curves for *L. monocytogenes* ATCC 19115 (×), LO28 (□), and 70-1700 (Δ) suspended in broth with pH 3.5 (HCl as acidulant) and $a_w$ 0.90 (NaCl as humectant) and incubated at 15°C.

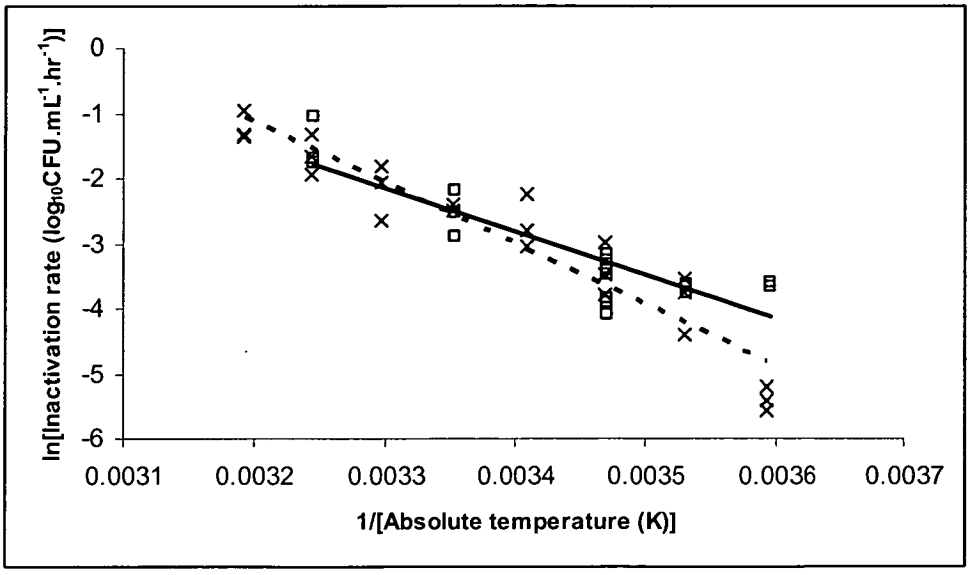


**Figure B.5.** Comparison of the Arrhenius models for the inactivation of *L. monocytogenes* suspended in broth with pH 3.5 (HCl as acidulant) and $a_w$ 0.90 (NaCl as humectant) for the current study (including addition inactivations at $a_w$ 0.93 and 0.95) (□) and for *L. monocytogenes* under the same conditions from the work by Zhang et al {Zhang, 2010 #416} (×). The data from both studies has had the inactivation rates at 45°C removed.

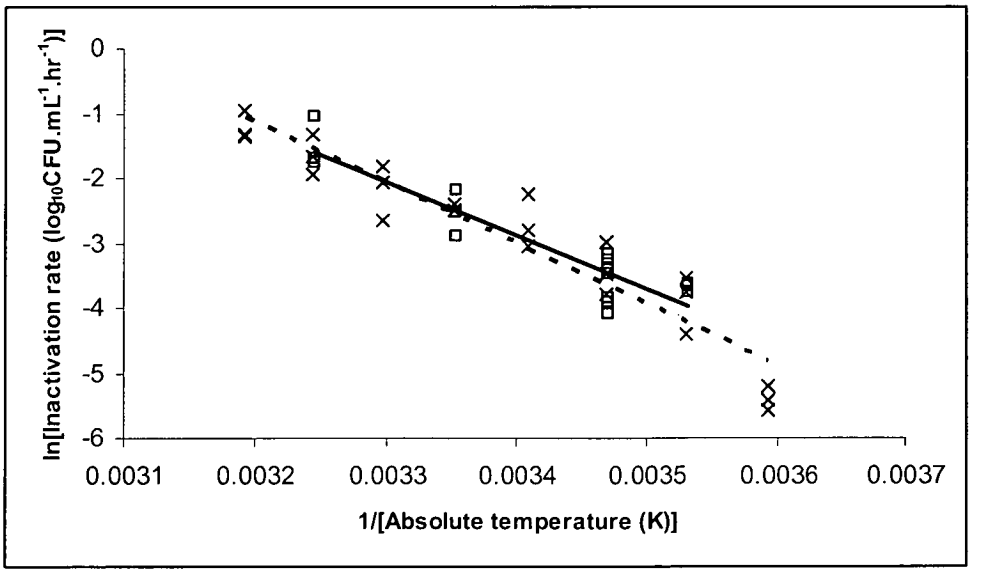


**Figure B.6.** Comparison of the same Arrhenius models in Figure B.5 but with the inactivation data at 5°C for this study removed.

## Appendix C: Proteomic Experiments

### C.1 Tryptic digestion solutions

A. 400mM Ammonium bicarbonate stock
Prepared by dissolving 1g of ammonium bicarbonate in 25ml of MilliQ water

B. 100nM Calcium Chloride
Prepared by dissolving 0.27g of anhydrous $CaCl_2$ in 25ml MilliQ water

C. Digest solution = 50mM Ammonium bicarbonate, 1mM Calcium chloride solution
Prepared by mixing 0.5ml of 400mM Ammonium bicarbonate stock (A) with 3.45ml of MilliQ water, and adding 40ul of 100mM Calcium chloride (B) solution.

D. 8M Urea, 100mM ammonium bicarbonate
2.7g of urea was placed in a 15ml centrifuge tube and 1.25ml ammonium bicarbonate stock (A) was added. The volume was made up to 5ml with MilliQ water.

E. Reducing agent: 50mM Dithiothreitol, 100mM ammonium bicarbonate
Prepared by dissolving 7.5mg of DTT in 250ul of ammonium bicarbonate stock (A) and 750ul of MilliQ water.

F. Alkylating reagent: 200mM iodoacetamide, 100mM Ammonium bicarbonate
Prepared by dissolving 36mg of iodoacetamide in 250ul of ammonium bicarbonate stock (A) and 750ul of MilliQ water. Photosensitive, therefore kept in the dark.

G. Trypsin solution (100ng/ul)
To 200ul of Digestion solution (C) 20ug of Promega sequencing grade modified trypsin (V511A) was added and kept at 4±1 °C in a refrigerator.

H. 10% Formic Acid

Prepared by adding 100ul of Formic Acid to 900ul of MilliQ water in an eppendorf tube.

### C.2 MudPIT running conditions

The Tryptic digests were analysed using multidimensional protein identification technology (MudPIT). The system used was a nanoflow triphasic MudPIT system consisting of a C18 capillary trap followed by a strong cation exchange resin (SCX) stage and, finally, an analytical C18 nano-column. The HPLC buffers used are detailed in Table 1.

50ul of Tryptic digest was loaded onto the C18 capillary trap at a flow rate of 35 ul/min using Buffer A. During sample loading the SCX and analytical columns were switched *via* a valve out of line of the C18 trap, with the trap being washed to waste to ensure salts and other non-peptide materials were not introduced into the mass spectrometer. After 10 minutes sample loading the SCX and Analytical columns were switched in-line with the capillary trap and the flow reduced *via* a splitter to 250 nl/min. A 5 step MudPIT gradient was performed as described in Table 2. The complete analysis time was 10.5 hours.

To ensure no sample contamination between runs occurred a 1.5 hour clean up procedure was performed. Table 3 details the gradient program utilised here.

The LTP Orbitrap mass spectrometer is a hybrid mass spectrometer, consisting of a 2D ion trap capable of low resolution MS/MS (LTQ part), and an electrostatic trap capable of performing high resolution, high mass accuracy analysis (Orbitrap component).

During the MudPIT analysis the mass spectrometer was operated in "data dependent" tandem mass spectrometry (MS/MS) mode. A "survey" scan performed in the electrostatic Orbitrap identified the possible parent peptide masses. The mass spectrometer then made a "data dependent decision" as to what peaks to analyse by tandem mass spectrometry, and the top 6 peaks were fragmented in the ion trap,

producing MS/MS data. This process took under 2 seconds to perform and was cycled continually throughout the MudPIT run. A typical LC-MS/MS produced between 10 000 and 20 000 MS/MS spectra to identify.

## Instrument

Mass Spectrometer: Thermo Electron, LTQ Orbitrap
HPLC: Thermo Electron, Surveyor HPLC

## Reagents

Formic Acid, Ammonium Acetate (MERCK, pro analysis grade)
Trifluoro Acretic Acid (MERCK, Uvasol)
Acetronitrle (J.T. Baker, BAKER ANALYSED, HPLC solvent)
Water (Reverse Osmosis, Deionised)

## Consumables

C18 Capillary Trap (Microme Bioresources, Peptide CapTrap)
SCX Trap (New Objective, IntegraFrit Column, 100um ID, 2.5cm, IFC10025-CX)
Analytical Column (New Objective, PicoFrit Column, 15um tip, 10cm, PFC7515-BI-10)

Table C.1. HPLC Buffers for MudPIT Analysis

| *Buffer* | *Composition* |
|---|---|
| A | 0.1% Formic Acid, 0.1% Triflouro Acetic Acid |
| B | 5% Acetronitrile + 0.1% Formic Acid |
| C | 90% Acetonitrile + 0.1% Formic Acid |
| D | 500mM Ammonium Acetate in 5% Acetonitrile |

Table C.2. 5 Step MudPIT HPLC Gradient Program

| *Step* | *Time (min)* | *%A* | *%B* | *%C* | *%D* |
|---|---|---|---|---|---|
| Loading | 0 | 100 | | | |
| | 10 | 100 | | | |
| 1 | 0 | | 100 | | |
| | 5 | | 100 | | |
| | 10 | | 85 | 15 | |
| | 70 | | 55 | 45 | |
| | 80 | | | 100 | |
| | 90 | | | 100 | |
| | 90.1 | | 100 | | |
| | 95 | | 100 | | |
| 2 | 0 | | 90 | | 10 |
| | 5 | | 90 | | 10 |
| | 5.1 | | 100 | | |
| | 20 | | 85 | 15 | |
| | 75 | | 75 | 25 | |
| | 125 | | 50 | 50 | |
| | 125.1 | | 100 | | |
| | 130 | | 100 | | |
| 3 | 0 | | 80 | | 20 |
| | 5 | | 80 | | 20 |
| | 5.1 | | 100 | | |
| | 20 | | 85 | 15 | |
| | 75 | | 75 | 25 | |
| | 125 | | 50 | 50 | |
| | 125.1 | | 100 | | |
| | 130 | | 100 | | |

| | | | | |
|---|---|---|---|---|
| 4 | 0 | 60 | | 40 |
| | 5 | 60 | | 40 |
| | 5.1 | 100 | | |
| | 20 | 85 | 15 | |
| | 75 | 75 | 25 | |
| | 125 | 50 | 50 | |
| | 125.1 | 100 | | |
| | 130 | 100 | | |
| | | | | |
| 5 | 0 | | | 100 |
| | 15 | | | 100 |
| | 15.1 | 100 | | |
| | 25 | 80 | 20 | |
| | 90 | 55 | 45 | |
| | 95 | | 100 | |
| | 115 | | 100 | |
| | 115.1 | 100 | | |
| | 130 | 100 | | |

Table C.3. Column Clean up Method

| *Step* | *Time (min)* | *%A* | *%B* | *%C* | *%D* |
|---|---|---|---|---|---|
| Cleaning | 0 | | | 100 | |
| | 10 | | | 100 | |
| | 11 | | | | 100 |
| | 19 | | | | 100 |
| | 20 | | 100 | | |
| | 50 | | | 100 | |
| | 75 | | | 100 | |
| | 76 | | 100 | | |
| | 90 | | 100 | | |